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"Galactose Dehydrogenase," "Nothing Dehydrogenase," and Alcohol Dehydrogenase: Interrelation

Cuatrecasas and Segal (*1*, *2*) recently suggested the existence of an interesting new pathway of galactose oxidation in liver tissue from various mammals. They also investigated the electrophoretic mobility of the first step of this pathway, "galactose dehydrogenase," in various species and during development. I have confirmed their experimental results but suggest that the enzyme responsible for the effects observed is really alcohol dehydrogenase (*Enzyme Nomenclature*: Alcohol:NAD oxidoreductase 1.1.1.1.), and the substrate is alcohol, contaminating galactose and other reagents.

I was initially struck by the similarity in the electrophoretic bands, reported by Cuatrecasas and Segal, to "nothing dehydrogenase." Shaw and Koen (*3*) had suggested that "nothing dehydrogenase" activity really represented alcohol dehydrogenase. Using the system described by Cuatrecasas and Segal I have been able to demonstrate that the band that they interpret as galactose dehydrogenase appears with equal intensity if galactose is omitted from the reaction mixture (Fig. 1). I have determined that commercially available hydrolyzed starch (Connaught) contains substantial amounts of alcohol, as measured with crystalline, alcohol-free, yeast or liver alcohol dehydrogenase and NAD (nicotinamide adenine dinucleotide). This alcohol can be removed simply by washing the starch in cold buffer; the activity appears in the washings and disappears after drying at 80°C. Phenazine methosulfate also contains small amounts of alcohol, as measured by the reduction of dichloroindophenol in the presence of crystallized liver alcohol dehydrogenase and NAD. However, only very weak bands or no bands of "nothing dehydrogenase" appear when electrophoresis is carried out on gels prepared from washed starch. These bands are greatly enhanced when alcohol is added to either the starch suspension or the staining mixture in a concentration of 2 m*M*.

Since the incorporation of galactose in the staining mixture failed to result in the appearance of a unique band, and because of my growing realization of the extent of contamination of high-grade reagent chemicals with alcohols, the supernatant fraction of crude homogenates and partially purified enzyme was further studied to investigate the possibility that "galactose dehydrogenase" was really alcohol dehydrogenase.

When galactose, NAD, buffer, and liver-homogenate supernatant are incubated together in the system described (*1*), an increase in optical density at 340 mμ is readily observed. The rate of reduction of NAD in my hands has been very similar to that reported (*1*). However, the following observations lead me to the conclusion that the observed reduction of NAD reflects the action of alcohol dehydrogenase on alcohol rather than the presence of a galactose dehydrogenase:

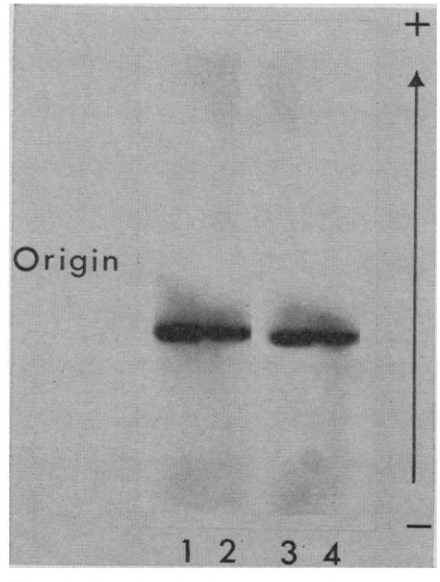

Fig. 1. Starch-gel electrophoresis of liver homogenate from mouse (channels *1* and *3*) and rat (channels *2* and *4*) by use of the system described for staining "galactose dehydrogenase" (*2*). Galactose was omitted from the staining mixture used for channels *3* and *4*.

1) I have found that the most highly purified, commercially available galactose preparations contain ample substrate for commercial, crystalline, horse-liver or yeast-alcohol dehydrogenase. One lot of galactose (essentially glucose-free; Sigma) was found to contain 0.6 mmole of substrate for alcohol dehydrogenase per mole of galactose, and the highest-grade galactose offered by Calbiochem contained 0.8 mmole of alcohol per mole of galactose.

2) The substrate for alcohol dehydrogenase that contaminates galactose is readily removed by drying a 1*M* solution of galactose at 80°C and reconstituting it with distilled water. No "galactose dehydrogenase" activity is found in liver when substrate subjected to this simple drying treatment is used, but there is no destruction of galactose, as measured with the galactose oxidase system (*4*).

3) The alcohol dehydrogenase activity of rat liver is readily partially purified by adsorption of extraneous proteins by treatment with diethylaminoethyl cellulose in 0.005*M* tris-HCl buffer, *p*H 8.8, and taking a 60- to 80-percent ammonium sulfate cut. During this approximately 20-fold purification, alcohol dehydrogenase shows activity with galactose that has not been subjected to drying at 80°C, but no activity with galactose so dried. The ratio of activity with untreated galactose to activity with ethanol remains unchanged throughout purification, and is the same as that found with crystalline horse-liver alcohol dehydrogenase.

4) The rate of reaction of the supernatant from rat liver with galactose is essentially identical with the rate found when the quantity of ethanol, equivalent to the amount of substrate for alcohol dehydrogenase contaminating the galactose, is used.

It seems apparent, therefore, that the "galactose dehydrogenase" activity of mammalian liver is really alcohol dehydrogenase activity acting on alcohols contaminating the reagents used. The highest-quality reagents commercially available to the biochemical laboratory today are contaminated with sufficient quantities of primary alcohols

to give misleading results in systems in which such alcohols react. My findings lead me to confirm the suggestion of Shaw and Koen that "nothing dehydrogenase" is alcohol dehydrogenase, and I consider it doubtful that the explanation sought for the anomalous oxidation of galactose by galactosemic subjects can be found in a liver galactose dehydrogenase.

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The alcohol dehydrogenase of vertebrate livers is an enzyme with broad substrate specificity. It has demonstrated activity toward several alcohols, aldehydes, and ketones (*1*, *2*), and has even shown some ability to transfer hydrogen in the absence of any known substrate—the "nothing dehydrogenase" activity (*3*); the reaction is NAD-linked (nicotinamide adenine dinucleotide) and is inhibited by sulfhydryl reagents. The molecule is strongly electropositive, as evidenced by rapid cathodal migration in starch-gel electrophoresis at approximately neutral *p*H. Its distribution in tissues of higher animals is limited mainly to the liver; an alcohol dehydrogenase has also been reported in retina (*4*), but has been shown to be a different molecule (*5*).

A galactose dehydrogenase was recently described, also mainly from vertebrate livers (*6*–*8*). Various similarities to the alcohol dehydrogenase (such as distribution, broad specificity, NAD-linkage, inhibition by sulfhydryl inhibitors, and starch-gel patterns) suggested that the galactose dehydrogenase activity might reside in liver alcohol dehydrogenase. Our evidence indicates the identity of liver galactose dehydrogenase and alcohol dehydrogenase.

Commercially purified equine-liver alcohol dehydrogenase (Sigma Chemical Company) was filtered through Biogel P-30 to remove ammonium sulfate. Fresh animal tissues were extracted by grinding in three volumes of distilled water in a glass homogenizer. After two freezings and thaws to rupture cell walls, the homogenate was centrifuged in the cold at 20,000*g* until the supernatant was clear. Assays for both alcohol and galactose dehydrogenase activities were performed in a Gilford model-2000 automatic spectrophotometer, production of reduced nicotinamide adenine dinucleotide (NADH) being measured at 340 mμ; ethyl alcohol, galactose, and glucose were used as substrates in equimolar amounts under zero-order conditions. Units of activity are expressed as micromoles of NADH produced per milligram of enzyme per minute.

Procedures for starch-gel electrophoresis were standard. Two different buffer systems were used for the gels: one of 0.005*M* phosphate, *p*H 6.6; the other, a mixture of 0.005*M* sodium succinate and 0.01*M* tris, *p*H 7.0. The buffer boxes contained the same buffers as did the gels, but at 20 times the strength. Vertical electrophoresis was done in the cold at a gradient of 6 volt/cm for 18 hours. The gels were then sliced horizontally, and half of the slices were incubated for alcohol dehydrogenase activity; the other half, for galactose dehydrogenase activity. An identical gel was prepared as a control, one half being incubated without added substrates. Incubation mixtures contained as substrate either 0.2*M* galactose or 0.1*M* ethyl alcohol, ethanol-free NAD^+, nitro blue tetrazolium, and phenazine methosulfate in 0.05*M* tris buffer (*p*H 7.0) to a final volume of 100 ml. For inhibition studies, *p*-hydroxymercuribenzoate at $10^{-4}M$ was added to the incubation mixtures.

Figure 1 shows a single starch gel that was halved, the left side being developed with ethyl alcohol as substrate; the other half, with galactose. The anodal direction is upward. Note that the patterns are mirror images; in each tissue, zones of alcohol dehydrogenase and galactose dehydrogenase demonstrate identical patterns. The samples shown are liver extracts of three deer mice (*Peromyscus*), purified horse-liver alcohol dehydrogenase, and horse-liver extract. The deer mice show two different patterns, which are presumed to reflect genetic variations. The difference in pattern between the purified alcohol dehydrogenase and horse liver may also be genetic; another preparation of alcohol dehydrogenase gave a pattern identical with that of this horse liver. Liver extracts of rat, house mouse, trout, and chicken yielded results similar to these, the last two showing weaker activities than did the mammals. All zones of activity found were produced by both substrates. The two activities cannot be compared quantitatively from the gels inasmuch as the galactose gel was incubated with a higher molarity of substrate and longer (2 hours versus 45 minutes) in order to develop the bands to a photographable level. Both activities were completely inhibited by *p*-hydroxymercuribenzoate. Kidney extracts of horse and rat showed weak alcohol dehydrogenase activity but no visible galactose dehydrogenase activity.

A control gel, run without added substrate, showed a pattern identical with those of gels containing substrate, but all bands were much weaker—the familiar "nothing dehydrogenase" ac-

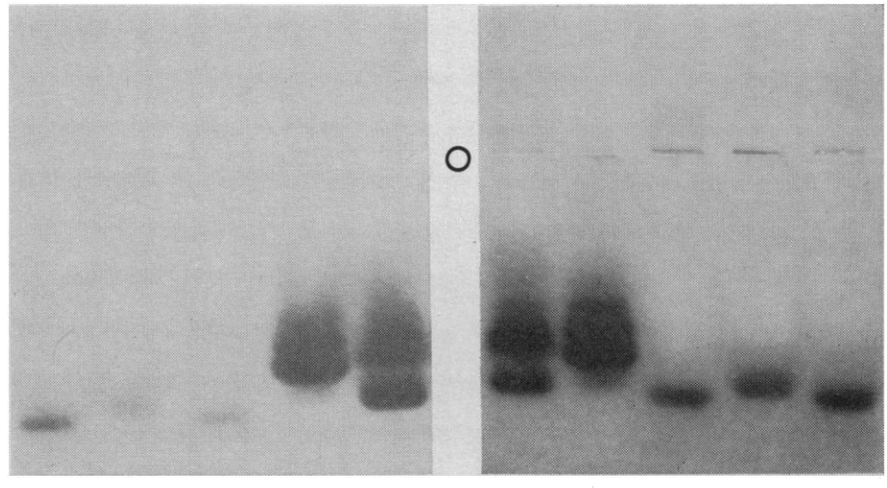

Fig. 1. Opposite halves of a single starch gel. Left half incubated with ethyl alcohol as substrate; right half, with galactose. Inner surfaces are shown, so that patterns are mirror images. Samples in left half, left to right, are liver extracts of three deer mice, purified horse-liver alcohol dehydrogenase, and horse-liver extract. Anodal direction is upward; origin is at *0*.

tivity of alcohol dehydrogenase (*3*). The results on several other substrates tested on starch gels, as estimated by visual inspection, indicated the following order of activity: hexyl alcohol > ethyl alcohol > benzyl alcohol > rhamnose > galactose > galactosamine > glucose = 0.

Quantitative studies of purified alcohol dehydrogenase, ethyl alcohol and galactose being used as substrates, demonstrated activities of 2749 and 233 units, respectively, per milligram of enzyme—an activity ratio of 11.8:1. With glucose, activity was so low as not to be consistently measurable; it was estimated to be of the order of 1/10th that of galactose.

With crude horse-liver extract, the activity ratio of alcohol to galactose was 7.3 : 1. Some reduction of NAD occurred in the blanks of both the pure ADH and the liver extract; the blanks contained all the reagents but substrate. Again, this is the "nothing dehydrogenase" activity and was subtracted from the results obtained with substrate when activities were computed.

Purification of galactose dehydrogenase from liver was not attempted, but comparison of the method described for its purification (*7*) with a standard method for purification of liver alcohol dehydrogenase (*9*) indicates that the former procedure would probably extract alcohol dehydrogenase.

The relatively higher activity of galactose dehydrogenase than of alcohol dehydrogenase in the liver extract, as compared with purified alcohol dehydrogenase, suggests either that alternate, multistep activities are operating in the liver extract, or that some inhibiting or activating substance is producing a differential effect. The latter case might imply different active sites for the two substrates. This problem requires further investigation.

The possibility of contamination of the purified alcohol dehydrogenase with a separate galactose dehydrogenase is not completely excluded. However, the fact that both activities occurred at identical positions under all gel conditions, including the genetically variant enzymes, renders this possibility unlikely.

It is of interest that galactose dehydrogenase activity of the alcohol dehydrogenase of maize has been reported (*10*), but it seems unlikely that the alcohol dehydrogenase molecules of higher plants and animals are structurally homologous (*1*).

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The physiologic role of liver alcohol dehydrogenase has long been a mystery, since most species do not ingest alcohol. The broad substrate specificity suggests that it may have other natural activities, and perhaps the oxidation of galactose is one of these. But this is not the main pathway for galactose metabolism, and whether it is a significant one remains to be determined. Beutler (*11*), who also has found that galactose dehydrogenase activity resides in alcohol dehydrogenase, suggests that the former may be due to contamination of galactose by alcohol, and that the galactose itself is not oxidized.

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Beutler suggests that the galactose dehydrogenase (GalDH) activity of mammalian liver is really due to alcohol dehydrogenase (ADH) acting on alcohols contaminating various reagents. This view is refuted by the fact that oxidation of galactose by a purified GalDH preparation results in stoichiometric conversion of galactose to galactonate or galactonolactone, or to both, and the fact that this oxidative reaction is reversible (*1*). It is difficult to see how oxidation of alcohol could result in formation of these products. Moreover, the oxidation of other sugars to the corresponding aldonic acids, the patterns of substrate specificity, and the effects of other sugars on the kinetics of the reaction (*2*) further substantiate this point.

Shaw and Koen make the interesting suggestion that the oxidation of galactose is catalyzed by ADH rather than by another enzyme. Beutler, however, indicated that there is no oxidation of galactose by ADH if the small amount of contaminating alcohol is removed from galactose.

The evidence of identity of the galactose and alcohol dehydrogenases is derived from *similarities* in very nonspecific parameters such as tissue distribution, broad substrate specificity, nicotinamide adenine dinucleotide–linkage, inhibition by sulfhydryl reagents, and starch-gel patterns of crude preparations. But it is much more important to reconcile the fact that there are some important *differences* between the two proteins: for example, ADH is routinely purified by chromatography with a carboxymethyl cellulose or DEAE column, with yields of 75 to 98 percent (*3, 4*), whereas GalDH is immediately and irreversibly destroyed by such treatment (*1*). Also, ADH is well known as a zinc metalloprotein whose activity is inhibited by EDTA and other chelating agents (*4*); GalDH, on the other hand, does not require metallic-ion cofactors and its activity is unaffected by NaCN, EDTA, or exhaustive dialysis against EDTA (*2*). Proof of the identity of these two proteins must not only explain such differences but also entail detailed studies (with pure proteins and substrates) of product identifications, kinetics of catalytic processes, and physical properties.

Although ADH is active with various different substrates, we know of no reports indicating that it can oxidize an aldose moeity to the corresponding aldonic acid or lactone. It has been suggested that liver GalDH belongs to a complex group of mammalian sugar-oxidizing enzymes (*2*), and it is indeed plausible that these could overlap in function some already well-recognized proteins. It seems more reasonable, however, that such common functions may occur with aldehyde dehydrogenases rather than with ADH's.

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