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Underestimation of alcohol dehydrogenase as a result of various technical pitfalls of the enzyme assay

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Recently, Crow *et al.* [1] summarized the rates of ethanol elimination in rats *in vivo*, obtained by several laboratories, which varied from 2.3 to 4.6 μmoles/g liver/min (average value: 3.32 ± 0.14 μmoles/g liver/min). Plapp [2] discussed the relationship of alcohol dehydrogenase (ADH) activities and *in vivo* oxidation rates in a major review. He concluded from a survey of the world literature that in several species the apparent ADH activity accounts for only 40–80 per cent of ethanol elimination rates *in vivo*. On the basis of this apparent ADH deficiency, some authors [3] have considered the microsomal ethanol-oxidizing system (MEOS) as a pathway which might quantitatively contribute to ethanol oxidation *in vivo*. But recently, Crow *et al.* [1] have convincingly demonstrated that in normal rat liver the ADH activities are as high as 5.05 ± 0.21 μmoles/g liver/min. Thus, the ADH present in liver can easily account for the ethanol disappearance rate *in vivo*. We have attempted to explore some possible mechanisms of an apparent underestimation of ADH as the reason for the frequently reported discrepancies between ADH activities and *in vivo* disappearance rates. Some authors [4] have previously described high unexplained background rates of the ADH assay (40 per cent of total rate) and others [5, 6] have recommended ethanol substrate concentrations up to 600 mM which had been shown by Krebs [7] and Dickinson and Dalziel [8] to be inhibitory for ADH in horse liver. These and other reports led us to investigate such technical problems as contamination of nicotinamide adenine dinucleotide (NAD) with ethanol as well as substrate inhibition of ADH by high ethanol concentrations and its pH dependency.

Materials and methods. Male Sprague–Dawley rats (250–500 g) fed on normal rat chow diet were used throughout the experiment. The following buffer systems were utilized for ADH measurements: (a) 0.5 M Tris buffer, pH, 9.5; (b) 85.5 mM sodium-pyrophosphate buffer/19.1 mM glycine, pH 9.0 [5]; (c) 12.5 mM imidazole buffer, pH 7.2; and (d) 0.5 M Tris buffer, pH 7.2 [1]. ADH activities were measured at 38° with a ZEISS PM 2-DL spectrophotometer.

Gas chromatography. NAD analysis was performed on a Hewlett–Packard Gas Chromatograph, model 5830A, using a 6 foot glass column packed with 0.1% SP-1000, 100/120 Carbopack C, G-3237, Supelco, Inc., Bellefonte, PA, U.S.A. The details of the gas chromatographic analysis program are shown in Fig. 3.

Chemicals. NAD was purchased from three different suppliers: (1) NAD ChromatoPure (TM) (NAD-PL), No. 2088, PL-Biochemicals, Inc., Milwaukee, WI, U.S.A.; (2) NAD, Grade III (NAD-Sigma), No. N-7004, Sigma Chemical Co., St. Louis, MO, U.S.A.; and (3) NAD, free acid Grade I (NAD-Boehringer), 100% pure, No. 15298, Boehringer Mannheim Biochemicals, Indianapolis, IN, U.S.A. Several lots from each supplier were tested, and the results were found to be consistent with our gas chromatographic analysis. 4-Methylpyrazole, a specific inhibitor for ADH, was obtained from Research Plus Laboratories, Inc., Denville, NJ, U.S.A. Alcohol dehydrogenase (EC 1.1.1.1) from yeast was purchased from Boehringer Mannheim GmbH, Federal Republic of Germany, No. 15420, lyophilysate, 400 units/mg protein. A rat liver 40,000 *g* supernatant fraction was prepared according to Crow *et al.* [1]. The ADH assays were started by addition of substrate (ethanol) except where stated otherwise.

Alcohol dehydrogenase and NAD contamination. During application of various assay systems for rat liver ADH, we observed a background rate which was clearly pH dependent. Figure 1 shows the significantly increased background rate at pH 9.5 (A) compared to the background rate at pH 7.2 (B). Addition of 5 mM ethanol further increased the rate of ethanol oxidation at pH 7.2 (B) but had no further effect on the rate observed at pH 9.5 (A). The use of Tris buffer, pH 7.2, instead of imidazole buffer (B) (not shown) did not alter the results.

4-Methylpyrazole (1 mM) inhibited ethanol oxidation completely in both (A) and (B) of Fig. 1. 4-Methylpyrazole also eliminated the background rate (Fig. 1C) at pH 7.2

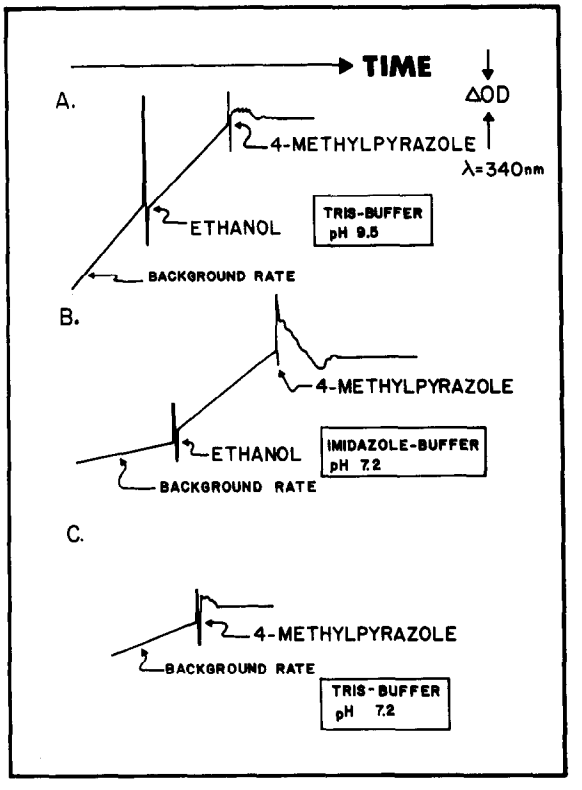

Fig. 1. Effects of various buffer systems of different pH values on the background rates of the ADH assay. A 40,000 *g* rat liver supernatant fraction was used as the enzyme source. The concentration of ethanol added as substrate (start of reaction) was 5 mM, NAD-PL was 2.8 mM, and 4-methylpyrazole was 1 mM; temperature, 38°.

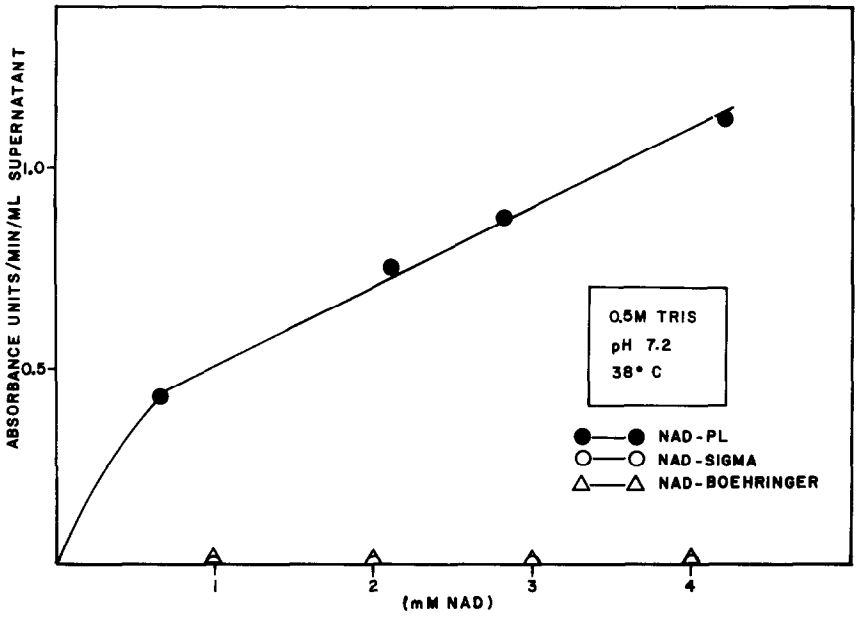

Fig. 2. Dependency of ADH background rates on increasing concentrations of NAD in the absence of additional substrate (ethanol).

in the absence of additional substrate (5 mM ethanol), suggesting that the background rate resulted from ADH. A subsequent search for possible ethanol contamination of buffers (TRIZMA-base, imidazole or glycine) was unsuccessful. Because we had exclusively utilized chromatographically pure NAD from PL-Biochemicals, Inc., we compared the effect of NAD from three commercial suppliers (NAD-PL, NAD Sigma, NAD-Boehringer) on the background rate of the ADH assay over an increasing NAD concentration range (Fig. 2). Under the specified conditions of 0.5 M Tris buffer, pH 7.2, 38°, and rat liver 40,000 *g* supernatant fraction as the enzyme source, in the absence of added substrate (ethanol), a linear increase in background rate was observed as the NAD concentration

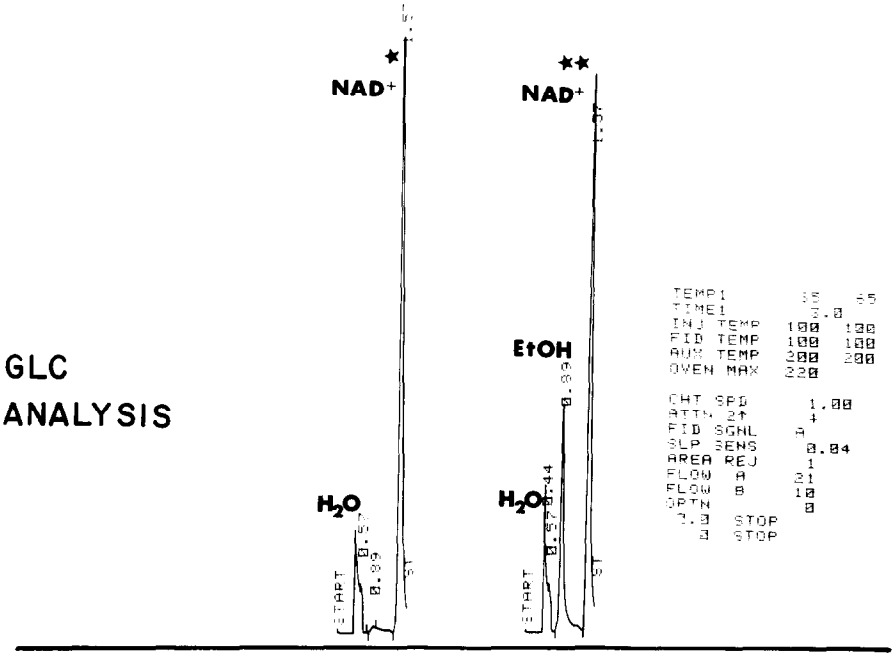

Fig. 3. Gas chromatographic analysis of NAD of various commercial sources. NAD* represents NAD-Sigma, and NAD** represents NAD-PL. Analysis of NAD-Boehringer (not shown) was identical to NAD-Sigma. A 30 mM NAD solution (0.1 μl) in distilled water was injected on the glass column. Details of the analytical program are printed above. EtOH = ethanol.

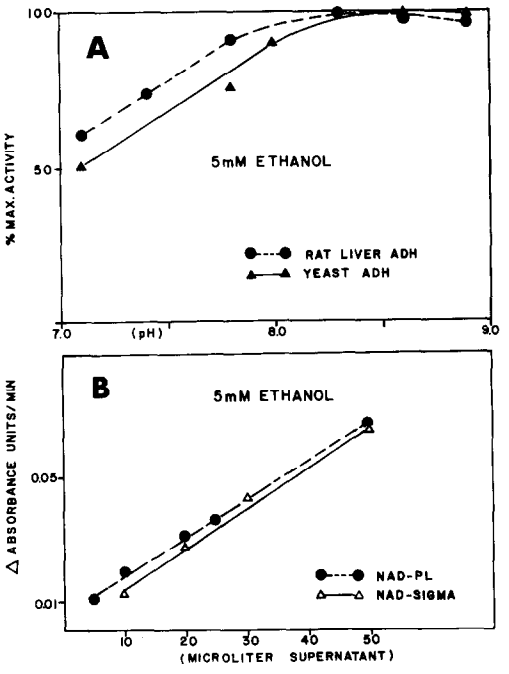

Fig. 4. Dependency of ADH activities of rat liver and yeast upon the pH of the assay system (A) as well as upon the amount of supernatant fraction added (B).

was increased from 1 to 4 mM. This effect could only be demonstrated with NAD from PL-Biochemicals, Inc., and was not observed with NAD-Sigma or NAD-Boehringer. Since this background rate (Fig. 1C) was sensitive to inhibition by 4-methylpyrazole, the contaminant was suspected to be ethanol itself.

Solutions of NAD from the three commercial suppliers were prepared and injected into a Hewlett–Packard Gas Chromatograph (Fig. 3) to measure adventitious ethanol. The sample of NAD-Sigma contained essentially no ethanol (neither did NAD-Boehringer) yet NAD-PL contained a significant peak at 0.89 min retention time which was identical to the ethanol standard (Fig. 3). The amount of adventitious ethanol in a cuvette containing 2.8 mM NAD-PL was 0.28 mM. This value is close to the apparent K_m value for ethanol of rat liver ADH (0.26–0.5 mM; Ref. 2).

Since increasing pH enhances ADH activity (Fig. 4A) at a fixed substrate concentration of 5 mM ethanol, contamination of NAD with alcohol would result in unacceptably large background rates at pH 9.5 (Fig. 1A). These, when subtracted from the rates after substrate (ethanol) addition, could falsely lower the overall calculated ADH activities by a large percentage. In contrast, if one determines ADH activities in the presence of V_{max} concentrations of ethanol (5 mM) plus any one of the NAD compounds by starting the reaction with addition of various amounts of supernatant enzyme, the inherent problem of NAD ethanol contamination is undetectable (Fig. 4B) since no initial background rate has been monitored. In the presence of NAD-PL, the final ethanol concentration in the cuvette would be 5.28 mM, instead of the 5 mM concentration when non-contaminated NAD is used.

Alcohol dehydrogenase, ethanol concentration and pH. Another problem which could significantly affect the proper determination of rat liver ADH is the concentration of substrate (ethanol) used. Although Dickinson and Dalziel [8] have previously reported substrate inhibition of horse liver ADH with increasing ethanol concentrations above 8 mM, the assay described for yeast by Bergmeyer [5] and Bernt and Gutmann [6] using 600 mM ethanol as substrate has occasionally been utilized [9]. Since this assay is the only ADH assay offered in Bergmeyer's book, investigators may be led to believe in its general application for any ADH system. But, as shown in Fig. 5, yeast ADH reached V_{max} rates only in the neighbourhood of 600 mM ethanol, whereas rat liver ADH was inhibited by 60 per cent at this ethanol concentration. This problem is emphasized in Fig. 6 where rat liver ADH and yeast ADH were compared at two different pH values and two different substrate levels, namely 5 mM and 600 mM ethanol. Rates at 5 mM ethanol have been set equal to 100 per cent as point of reference, but the absolute rate with 5 mM ethanol at pH 9.0 was approximately 40–50 per cent higher than at pH 7.2.

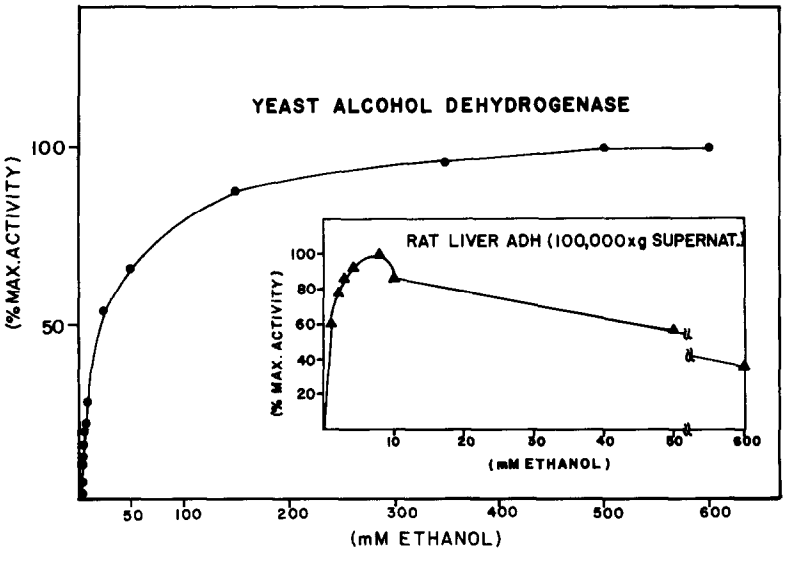

Fig. 5. Substrate concentration curves of ADH activities from yeast and rat liver (100,000 *g* supernatant fraction; insert). Assay conditions were: 0.5 M Tris buffer, pH 7.2; 2.8 mM NAD-Sigma; temperature 38°.

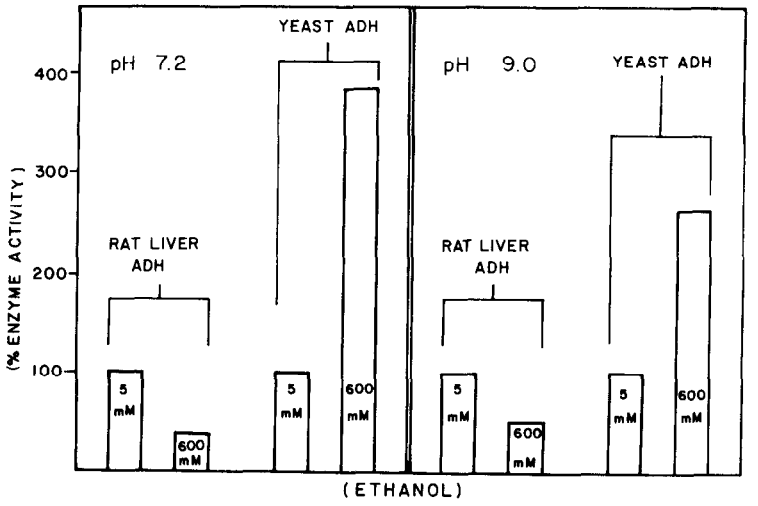

Fig. 6. Comparison of low (5 mM) and high (600 mM) ethanol concentrations on the activities of rat liver and yeast ADH at two different pH values (pH 7.2 vs pH 9.0). For pH 7.2, the assay of Crow *et al.* [1] was used. For pH 9.0, the assay of Bergmeyer [5] was applied. Rates at 5 mM ethanol were set equal to 100 per cent activity. At pH 9.0, the absolute ADH activity at 5 mM ethanol was enhanced as compared to pH 7.2 (see also Fig. 4A).

Although some investigators [9] determined rat liver ADH activities with 600 mM ethanol as substrate [5, 6], their rates (4.37 ± 0.77 μmoles/g liver/min) were only moderately below the range of the maximum rates recorded by Crow *et al.* [1]. The reason for the moderate inhibition is that two counteractive events almost completely masked the inherent problem of substrate inhibition. At pH 7.2, 600 mM ethanol inhibited rat liver ADH by almost 60 per cent (Figs. 5 and 6), but by choosing pH 9.0 [5, 6] for the assay rat liver ADH was enhanced (Fig. 4A) by *ca.* 40 per cent compared to pH 7.2. The subsequent addition of 600 mM ethanol inhibited this rate by approximately 40–50 per cent (Fig. 6). The end result was a 10–20 per cent inhibition of ADH activity when compared to assays performed at pH 7.2 and 5 mM substrate concentration. These interactions of pH and substrate concentration explain the values reported previously [9]. If one chooses to perform the ADH assay of Bergmeyer [5] and Bernt and Gutmann [6] at the physiological pH of 7.2 instead of pH 9.0 as recommended by these authors, rat liver ADH activites will be underestimated by at least 60 per cent.

In summary, two major causes of apparent underestimation of rat liver ADH, which has been experienced by us and seems to be evident from the published literature, have been explored. First, high pH-dependent background rates can result from contamination of commercially available NAD. Second, the basic assay principles recommended for yeast ADH by Bergmeyer [5] and Bernt and Gutmann [6] are not directly applicable to rat liver ADH because of strong substrate inhibition observed at 600 mM ethanol. This effect becomes most prominent if the assay system is altered toward a more physiological pH value of 7.2. Unaware of these consequences, another major handbook of enzymology [10] recommends an assay especially for rat liver ADH with substrate concentrations between 500 and 600 mM ethanol in the cuvette. The great number of low ADH values published in the literature suggests that underestimation on the basis of technical problems may be more prevalent than previously appreciated. Since proper measurements of ADH according to the method by Crow *et al.* [1] will yield enzyme activities greater than the rate of ethanol disappearance *in vivo*, a quantitative contribution of MEOS to ethanol removal *in vivo* in the normal rat is unlikely.

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Liver-Gastroenterology Units, The University of Texas Health Science Center at Dallas, Dallas, TX 75235, U.S.A.

RAINER N. ZAHLTEN
C. JAN JACOBSON
MICHAEL E. NEJTEK

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