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Effect of Temperature on the Assay of Hepatic Alcohol Dehydrogenase[1] (39792)

EDWARD A. CARTER AND KURT J. ISSELBACHER[2]

Department of Medicine, Harvard Medical School, and Gastrointestinal Unit, Massachusetts General Hospital, Boston, Massachusetts 02114

Introduction. During the course of studies on the activity of hepatic alcohol dehydrogenase (ADH) in rats with chronic alcohol ingestion, we examined the effect of increasing the assay temperature on the ADH activity of hepatic 5000*g* supernatant fractions. These fractions were prepared in the presence or absence of Triton X-100, a detergent which had previously been reported to alter the temperature optima of some enzyme systems (1). Preliminary results suggested that there was an enhanced ADH activity of rat liver 5000*g* supernatant (made without Triton X-100) when the assay temperature was increased. The present report describes the effect of increased assay temperature on hepatic and yeast ADH activities. The effect of Triton X-100 on hepatic ADH will be reported in a subsequent communication.

Methods. ADH activity was measured in a Gilford recording spectrophotometer equipped with a circulating water bath as described previously (2). The buffer and cofactor, kept at 25°, were mixed with the enzyme (kept on ice) and preincubated for 10 min in the spectrophotometer with the circulating water bath set at the appropriate assay temperature; ethanol or water (both at 25°) was then added to the test or control cuvette, respectively. Measurement of NADH formation was made during the first minute after the ethanol or water was mixed with the preincubated buffer, cofactor, and enzyme. Temperatures were recorded in the assay cuvette at the end of the reaction, 30 sec after insertion of a thermometer. Protein was determined by the method of Lowry *et al.* (3).

Fresh liver from female Sprague-Dawley rats or fresh horse liver was minced and homogenized in 0.25 *M* sucrose (10 vol/g wet weight). The homogenates were spun at 9000*g* for 30 min and the resulting supernatant was spun at 105,000*g* for 1 hr. The specific ADH activity of these supernatants varied somewhat; the averages, respectively, for rat and horse liver were 15 and 500 nmole of NADH formed/mg of protein/min at 25°.

Purified horse liver ADH was obtained as a lyophilized powder (Worthington Biochemical Co., N.J.; sp act = 2.3 μmole of NADH formed/mg of protein/min at 25°); purified yeast ADH was obtained as a crystalline suspension (Boehringer Mannheim, N.Y.; sp act = 200 μmole of NADH formed/mg of protein/min at 25°). Both preparations were diluted in 100 m*M* sodium phosphate buffer, pH 7.4, before use. Purified human liver ADH (sp act = 0.1 μmole of NADH formed/mg of protein/min at 25°) was isolated as described by Von Wartburg *et al.* (4).

Rat blood was obtained by exsanguinating female rats under ether anesthesia via the abdominal aorta with heparinized syringes; plasma was obtained by centrifugation at 600*g* at 4° for 10 min. In some experiments rats were given carbon tetrachloride (2 ml/kg, via intragastric tube followed by 2 ml of 0.45% NaCl). In other studies animals were fed a liquid diet containing 5% ethanol for 10 days, the controls receiving the same diet with glucose isocalorically replacing the ethanol (5). Plasma from these animals was dialyzed against 1000 volumes of water to remove ethanol and frozen for subsequent ADH assays. Freezing plasma for 3 days did not appear to affect ADH activity.

In some experiments using rat 105,000*g* supernatant or purified horse liver ADH, the reaction at 25 and 60° was allowed to

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[2] Send reprint requests to Kurt J. Isselbacher, M.D., Gastrointestinal Unit, Massachusetts General Hospital, Boston, Mass. 02114.

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proceed for 10 min, after which the NADH was extracted with KOH and measured fluorometrically as described elsewhere (6). In other experiments, the substrate was [1-^{14}C]ethanol (10 μCi/mmole) and the reaction was allowed to proceed for 1 hr. The liberated [^{14}C]acetaldehyde was trapped with 2,4-dinitrophenylhydrazine and identified by subsequent descending chromatography followed by radioactive counting (7).

Results. Enhancement of hepatic ADH activity by elevation of the assay temperature. ADH activity, measured as NAD reduction with ethanol as the substrate, was found to be markedly enhanced by an increase in the assay temperature (Fig. 1). The ADH activity of rat liver 105,000*g* supernatants was stimulated eightfold by raising the assay temperature from 25 to 60°; a less dramatic increase (threefold) was observed with horse liver 105,000*g* supernatant fraction. Purified human liver ADH activity was 2.5-fold greater at 55° (Fig. 1). In other studies we observed that purified horse liver ADH activity was twice as great at 60 compared to 25°. It should be noted that the stimulation of the hepatic ADH was not observed if the heated samples were allowed to cool to room temperature before ethanol was added and the assay was then carried out at 25°. In comparison to these results, purified yeast ADH activity was not increased but, in fact, was completely inhibited at temperatures greater than 45°.

The products of the reaction with ethanol as substrate plus rat liver supernatant or purified horse liver ADH and NAD were determined as described in Methods. At both 25 and 60° these products were identified as NADH and acetaldehyde.

1,10-Phenanthroline, which inhibits ADH by binding the zinc of the enzyme (8), decreased the ADH activity of the rat liver supernatant by 64% and that of the purified horse liver ADH by 70% at 25° (Table I). At 60°, this agent caused 89 and 77% inhibition of rat supernatant and purified horse liver ADH, respectively. Silver nitrate and *p*-chloromercuribenzoate, which interact with the sulfhydryl groups of ADH (9), completely inhibited the activity of the rat supernatant fraction and purified horse liver ADH at both 25 and 60°.

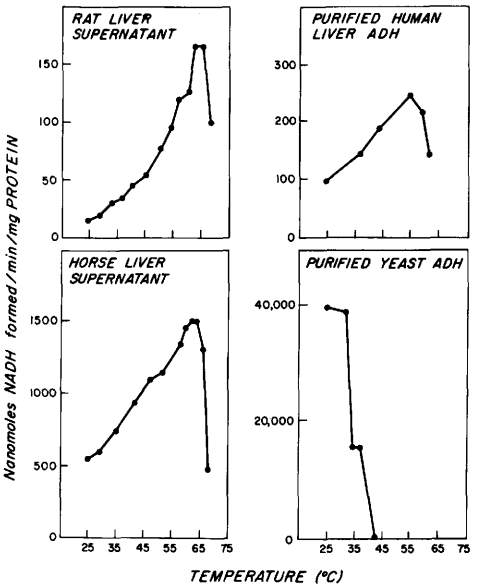

FIG. 1. Effect of assay temperature on alcohol dehydrogenase (ADH) activity. Assay mixture of 3.3 ml consisted of 50 m*M* glycine–sodium hydroxide buffer, pH 9.6, 50 m*M* ethanol, 1.5 m*M* NAD, and 0.1 ml of ADH-containing fraction. Rat or horse 105,000*g* liver supernatant contained 1 mg and 0.1 mg of protein, respectively, per 0.1 ml. Purified human liver or yeast ADH contained 0.1 mg and 0.03 mg of protein/0.1 ml. All assay mixtures were preincubated for 10 min at the various temperatures indicated. The results are the means of three or more experiments.

All the ADH preparations tested in this study (purified horse and human ADH, rat liver supernatant, and purified yeast ADH) were able to reduce NAD with 2-butanol and oxidize NADH with 2-butanone at 25° (Table II). With the exception of the yeast enzyme, elevating the assay temperature to 60° stimulated ADH activity in *both* the forward and reverse directions to the same degree (horse liver, twofold; human, fourfold; rat, threefold).

The kinetic parameters of the ADH reaction at 25 and 60° were measured and plotted according to Lineweaver and Burk. A typical plot for the rat liver supernatant is shown in Fig. 2. Here the V_{max} was increased sixfold at 60 compared to 25°. The K_m increased by about 50%. We examined the V_{max} and K_m values for both the rat liver supernatant and purified horse liver ADH at 25 and 60° over a wide range of pH levels (Table III). The increase in assay tempera-

TABLE I. EFFECT OF INHIBITORS ON LIVER ADH ACTIVITY AT 25 AND 60°.[a]

Additions	Rat liver 105,000g supernatant				Purified horse liver ADH			
	25°		60°		25°		60°	
	Units	Inhibition (%)	Units	Inhibition (%)	Units	Inhibition (%)	Units	Inhibition (%)
None	11.2	0	76.6	0	1836		3601	0
1,10-Phenanthroline (2 m*M*)	4.0	64	8.8	89	543	70	846	77
Silver nitrate (1 m*M*)	0	100	0	100	0	100	0	100
p-Chloromercuribenzoate (1 m*M*)	0	100	0	100	0	100	0	100

[a] Assay conditions as in Fig. 1. Horse liver ADH concentration was 0.066 mg of protein. All assay mixtures were preincubated for 10 min at the temperatures indicated. Rates are expressed as nanomoles of NADH formed per milligram of protein per minute at the temperatures indicated.

TABLE II. EFFECT OF TEMPERATURE ON VARIOUS ADH PREPARATIONS USING EITHER 2-BUTANOL OR 2-BUTANONE AS SUBSTRATE.

ADH source	ADH activity[a]					
	Cofactor = NAD			Cofactor = NADH		
	Temperature			Temperature		
	25°	60°	Change (%)	25°	60°	Change (%)
Horse liver (purified)	798.4	1741.9	+118	232.8	517.1	+122
Human liver (purified)	147.5	607.0	+312	59.5	254.5	+326
Rat liver (105,000g supernatant)	8.0	27.1	+239	2.3	7.9	+243
Yeast (purified)	141.2	0	−100	15.4	0	−100

[a] Assay mixture of 3.3 ml consisted of 50 m*M* glycine-sodium hydroxide buffer NaOH, pH 9.6, 0.294 mg of protein-purified yeast ADH, 0.066 mg of protein-purified horse liver ADH, 0.1 mg of protein-purified human liver ADH, or 0.1 ml of rat liver 105,000g supernatant (1 mg of protein), 34 m*M* 2-butanol or 2-butanone, and 0.46 m*M* NAD or 0.14 m*M* NADH. Rates are expressed as nanomoles of NADH formed or oxidized per milligram of protein per minute at the temperatures indicated.

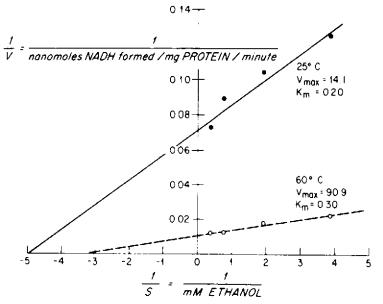

FIG. 2. Lineweaver-Burk plot of ADH activity of rat liver 105,000g supernatant at 25 and 60°. Assay conditions as in Fig. 1 except that the ethanol concentration was varied from 0.2 to 2 m*M*.

ture was always associated with large increases in V_{max} but only small alterations in K_m.

Effect of temperature on steroid oxidation by ADH. The effect of assay temperatures upon the reduction of 17-β-hydroxy-5-β-androstan-3-one (5β-DHT) by purified horse liver ADH was examined using the assay conditions of Pietruskzo *et al.* (10), which consisted of 100 m*M* sodium phosphate buffer, pH 7.4, horse liver ADH (0.5 mg of protein), 0.14 m*M* NADH, and 5β-DHT (0.01 mg) in a final volume of 3.21 ml. Increasing the assay temperature from 25 to 60° led to a fivefold increase in the reduction of 5β-DHT with NADH (i.e., from 13.9 to 63.3 nmole of NADH oxidized/mg of protein/min).

Assay of plasma ADH. At 25° both normal rat and human plasma failed to reduce NADH with ethanol (Table IV). However, at 50° definite activity was detected, rat plasma being more active than human plasma. Under the conditions described above, the products of the reaction at 50° (i.e., NADH and acetaldehyde) were extracted and identified. Studies of rat plasma 1.5 and 3 hr after the administration of CCl_4 showed 200 and 300% increases respectively, in plasma ADH activity (Fig. 3). In addition, compared to control animals, chronic ethanol-fed rats showed a 60% in-

TABLE III. KINETIC PARAMETERS OF THE ALCOHOL DEHYDROGENASE (ADH) REACTION MEASURED AT 25 AND 60°.

	Horse liver ADH[a]				Rat liver 105,000g supernatant ADH			
	V_{max}[b]		K_m[c]		V_{max}		K_m	
pH	25°	60°	25°	60°	25°	60°	25°	60°
7.6	1176	2174	26.0	35.0	11	58	3.3	4.3
8.6	2326	4348	7.1	7.1	11	50	3.3	4.3
9.6	2500	5000	1.0	1.1	14	91	0.2	0.3

[a] Assay conditions as in Fig. 1 except that the ethanol concentration was varied from 0.1 to 10 mM. V_{max} and K_m were calculated from Lineweaver-Burk plots.
[b] Nanomoles of NADH formed per milligram of protein per minute.
[c] mM.

TABLE IV. ADH ACTIVITY OF RAT AND HUMAN PLASMA.

	ADH activity[a]	
Plasma	25°	50°
Rat (6)	0	59.0
Human (2)	0	11.2

[a] Assay mixture as in Fig. 1 except that the enzyme source was 0.5 ml of plasma. Rates are expressed as nanomoles of NADH formed per milliliter of plasma per 10 min. Numbers in parentheses refer to numbers of samples assayed.

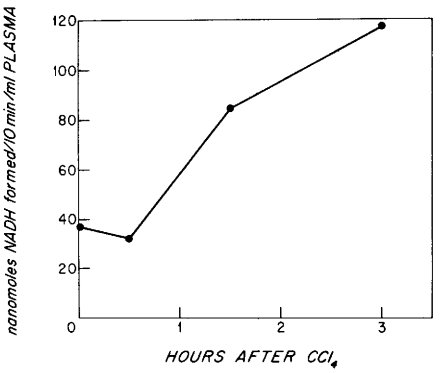

FIG. 3. Effect of carbon tetrachloride treatment of rats on plasma ADH activity. Assay conditions as in Table III except that the plasma was from nonfasted rats pretreated with carbon tetrachloride (2 ml/kg intragastrically, followed by 2 ml of 0.45% sodium chloride). The ADH assay was run at 50°. Three animals were used for each time point.

crease in their plasma ADH activity (i.e., 8.9 vs 14.1 nmole a NADH formed/ml of plasma/10 min).

Discussion. It is well recognized that a temperature elevation can have at least two effects on an enzymatic reaction: (i) an increase in the initial velocity until a maximal velocity is achieved, followed by (ii) a decrease in the rate with increasing temperature. In the present report we examine the effect of temperature on activities of liver and yeast alcohol dehydrogenase (ADH). The properties of ADH, including its substrate specificity and pH optimum have been reviewed by Von Wartburg (11).

A wide range of agents and treatments has been used to inhibit ADH (11), but little attention has previously been devoted to methods for *enhancing* ADH activity. ADH activity can be increased by using the 3-acetylpyridine analog of NAD because the reduced form of this analog dissociates from the enzyme much faster than NADH (12). The activity of ADH can also be enhanced by substances such as methyl picolinimidate, presumably by modifying the amino groups at the active sites (13). Imidazole, an inhibitor of ADH, can stimulate ADH at high NAD concentrations apparently by causing instability of the enzyme-NADH complex (14). The effect of chronic ethanol feeding on hepatic ADH activity is still unclear; some studies have shown stimulatory effects (15), while others have shown either no changes or even inhibition (11).

The results of the present study indicate that hepatic ADH can be stimulated by elevating the assay temperature to 50–60°. While the degree of stimulation of liver ADH varied somewhat between species (i.e., rat, horse, man), yeast ADH was completely inhibited at these temperatures. Stimulation of hepatic ADH did not occur if the samples were preheated and then al-

lowed to cool prior to ethanol addition and the assay was carried out at 25°. It is noteworthy that Wiseman and Williams (16) reported that preincubation of horse liver ADH at 43° for 50 min resulted in an approximately 60% reduction of enzyme activity and that a 30-min preincubation period produced 25% inactivation. They also observed a 60% loss of yeast ADH activity after a 20-min preincubation at 43° (16). However, these workers allowed the temperature of their preparations to decrease to 30° before assaying for ADH activity. In the present report, the preparations were heated and assayed at the same temperature and preincubations were for 10 min. It was noted that, if preincubation was continued beyond 10 min, the ADH activity started to decrease progressively.

The present results suggest that the stimulating effect of the increase in temperature involved changes in the function of the enzyme. Thus, when 2-butanol and 2-butanone were used as substrates, there was equal enhancement of ADH activity in both the forward and reverse directions. Furthermore, elevation of the assay temperature to 60° changed the kinetics of the ADH reaction with two- to fourfold increases in V_{max} and minimal changes in K_m over a wide pH range (7.6–9.6). However, the exact mechanism of the temperature effect, such as alterations of the active site or conformational changes, remains to be determined.

ADH activity has not been previously detected in normal plasma (17, 18) when assayed at 25°. However, as shown in the present study, plasma ADH activity could be demonstrated when the assay was carried out at 50°. This activity was found to increase when liver damage was produced by CCl_4 and also in association with chronic alcohol feeding. Mezey *et al.* (17) observed alcohol dehydrogenase in plasma (at 25°) of rats receiving this same dose of carbon tetrachloride 4 hr after administration of the toxin, while we found increases as early as 1.5 hr (at 50°). The appearance of plasma ADH activity (as 25°) has been suggested as a test for intrahepatic bile stasis as well as hepatocellular necrosis (17, 18). It is possible that the increased sensitivity obtained by measuring plasma ADH activity at 50° may make this assay a useful clinical tool for studying patients with liver injury.

Summary. An increase in the assay temperature of hepatic alcohol dehydrogenase (ADH) activity, from 25 to 50–60°, with ethanol as the substrate, led to a stimulation of enzyme activity. The stimulation of the hepatic ADH (not observed with yeast ADH) was reversible if the heated samples were allowed to cool to room temperature and then assayed at 25°. Only at the elevated temperature (50–60°) could ADH activity be detected in normal rat or human plasma. The increased sensitivity resulting from thermal activation of ADH may prove useful for studying the enzyme in crude preparations, measuring ADH activity with substrates normally providing low activity (e.g., 17-β-hydroxy-5-β-androston-3-one), or measuring serum ADH activity in patients with various types of liver injury.

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