Pharmacology Biochemistry & Behavior, Vol. 18, Suppl. 1, pp. 223–227, 1983. © Ankho International. Printed in the U.S.A.

Relationship Between Kinetics of Liver Alcohol Dehydrogenase and Alcohol Metabolism[1]

WILLIAM F. BOSRON,[2] DAVID W. CRABB AND TING-KAI LI

Departments of Medicine and Biochemistry, Indiana University School of Medicine and Veterans Administration Medical Center, Indianapolis, IN 46223

BOSRON, W. F., D. W. CRABB AND T.-K. LI. *Relationship between kinetics of liver alcohol dehydrogenase and alcohol metabolism.* PHARMACOL BIOCHEM BEHAV **18:** Suppl. 1, 223–227, 1983.—Since alcohol dehydrogenase (ADH) catalyzes the rate-limiting step for ethanol metabolism, knowledge of the steady-state kinetics of ADH in liver is fundamental to the understanding of the pharmacokinetics of ethanol elimination. Accordingly, we have determined the kinetic properties of purified ADH isoenzymes in rat and human liver. At low ethanol concentrations, rat liver ADH obeys the Theorell-Chance mechanism and the equation predicts that activity *in vivo* is limited below V_{max} mainly by NADH inhibition. At ethanol concentrations above 10 mM, substrate inhibition, consistent with the formation a dead-end ADH-NADH-ethanol complex, also becomes a rate-limiting factor. ADH activity, calculated from this equation and the concentrations of substrates and products present in liver during ethanol oxidation, agrees well with ethanol elimination rates measured *in vivo*. With human liver ADH, large differences are observed in the kinetic properties of 5 homodimeric isoenzymes: $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ exhibit negative cooperativity for ethanol saturation, while $\alpha\alpha$, $\beta_1\beta_1$ and $\beta_{Ind}\beta_{Ind}$ obey Michaelis-Menten kinetics. At pH 7.5, K_m values for ethanol and V_{max} values range from 0.048 mM and 9 min^{-1} for $\beta_1\beta_1$ to 64 mM and 560 min^{-1} for $\beta_{Ind}\beta_{Ind}$, respectively. Therefore, individuals with different ADH phenotypes should display different ethanol elimination profiles.

Alcohol dehydrogenase Alcohol metabolism Steady-state kinetics Isoenzymes

THE various pharmacologic, addictive and pathologic consequences of alcohol consumption are directly related to the biochemical properties of ethanol and its metabolic by-products, and to the rate of metabolism of these compounds. The rate-limiting step for ethanol metabolism is its oxidation to acetaldehyde by alcohol dehydrogenase (ADH) in liver [18]. It has been proposed that the steady-state kinetics of ADH can serve as a quantitative base for predicting the pharmacokinetics of alcohol elimination *in vivo* [9, 20, 23]. In order to test this hypothesis, the content of ADH in liver, the concentration of substrates and products of the enzyme *in situ* during alcohol metabolism and the steady-state kinetic equation and constants for purified ADH must be determined. The kinetic properties of ADH have proven difficult to determine in the past, because methods for purification and stabilization of enzyme for such studies have become available only recently. By use of affinity and ion-exchange chromatography, we have now purified ADH from rat liver and human liver and determined the steady-state kinetic properties of these enzymes (Crabb *et al.*, submitted for publication; Bosron *et al.*, submitted for publication).

This communication compares the alcohol oxidation rates calculated from the steady-state kinetic equation for rat liver ADH with that measured *in vivo* in fed rats [20]. The effects of substrate inhibition by ethanol and product inhibition by NADH on the calculated rates for rat liver ADH are evaluated. Similar calculations of ethanol metabolic rates in man from kinetic data for human liver ADH are difficult to perform because there are as many as 16 different isoenzymes, many of which exhibit widely different kinetic properties [4,18]. However, based on the findings summarized herein, it would appear that the 2 to 3-fold variation in ethanol elimination rate observed among individuals [16] can be readily explained by the differences in kinetic properties of the isoenzymes that are produced by either one of two polymorphic gene loci [4,24].

STEADY-STATE KINETICS OF RAT LIVER ADH AND REGULATION OF ETHANOL ELIMINATION *IN VIVO*

Rat ADH has been purified approximately 150-fold from homogenate-supernatants of liver by chromatography on DEAE-cellulose and CapGapp-Sepharose [10]. The purified enzyme exhibits a single band on SDS-polyacrylamide gel electrophoresis and has a specific activity of 1.8 μmol min^{-1} mg^{-1} protein when assayed with 10 mM ethanol and 2.4 mM

[1]Supported by U. S. Public Health Service grants AA-04307 and AA-02342.

[2]Requests for reprints should be addressed to Dr. William F. Bosron, 421 Emerson Hall, Indiana University Medical Center, 545 Barnhill Drive, Indianapolis, IN 46223.

NAD^+ at pH 10, 25°. The purification can be accomplished in about 6 hours and it represents a significant improvement over other procedures, since the yield is 2 to 5-fold higher than that reported previously [1,21]. The enzyme activity can be stabilized in solution for several days by the addition of the sulfhydryl-reducing agent, dithiothreitol. Enzyme stabilization has allowed a variety of steady-state kinetic experiments to be performed on single enzyme preparations.

Multiple enzyme forms from rat liver have been identified by starch gel electrophoresis [10,21]. Since some of the ADH isoenzymes from horse and human liver differ substantially in their kinetic properties [18,22], the two major rat liver forms were separated by chromatography on CM-cellulose and their kinetic properties determined ([11], Crabb *et al.*, submitted for publication). Both forms were found to exhibit similar kinetic properties. Accordingly, the steady-state kinetic experiments were performed with the CapGapp-Sepharose-purified enzyme that contains both forms.

Product and pyrazole inhibition studies [11] yielded data consistent with the Theorell-Chance mechanism and substrate inhibition occurring through the formation of a dead-end ternary ADH-NADH-ethanol complex [13,26]. The rate equation for this mechanism is:

$$v = \frac{V_F[A][B] - V_f\frac{[P][Q]}{K_{eq}}}{K_{ia}K_b + K_b[A] + K_a[B] + [A][B] + \frac{K_{ia}K_bK_q[P]}{K_pK_{iq}} + \frac{K_{ia}K_b[Q]}{K_{iq}} + \frac{K_bK_q[A][P]}{K_{iq}K_p} + \frac{K_{ia}K_b[P][Q]}{K_pK_{iq}} + \frac{K_a[B][Q]}{K_{iq}} + \frac{K_{ia}K_b[B][Q]}{K_iK_{iq}} + \frac{K_a[Q][B]^2}{K_iK_{iq}} + \frac{[A][B]^2}{K_i}} \quad (1)$$

The rate constants in Equation 1 are named according to Wratten and Cleland [26] and their values were determined through statistical fits [8] of data from product inhibition experiments. The Michaelis constants are: $K_a(NAD^+) = 33\ \mu M$, $K_b(\text{ethanol}) = 0.48$ mM, $K_p(\text{acetaldehyde}) = 37\ \mu M$, $K_q(NADH) = 4\ \mu M$; the inhibition constants are: $K_{ia}(NAD^+) = 58\ \mu M$, $K_{iq}(NADH) = 0.9\ \mu M$, K_i(ethanol substrate inhibition) = 170 mM; and $K_{eq} = 3.5\times10^{-4}$. These values agree well with the data of Hanozet *et al.* [14] but not with the Michaelis and inhibition constants for NAD^+ and NADH reported previously for ADH in unpurified homogenate-supernatants [9] nor with the Michaelis constant for ethanol reported previously for a purified preparation of rat liver ADH [1].

In order to examine whether ethanol elimination rates *in vivo* [19] can be predicted by Equation 1, we measured ethanol, acetaldehyde, lactate and pyruvate concentrations in freeze-clamped livers of fed rats metabolizing alcohol [20] (Table 1). The NAD^+/NADH ratio in Table 1 was calculated from the lactate/pyruvate ratio and free NAD^+ concentration was assumed to be 0.5 mM [7]. Free NADH was calculated from this value and the NAD^+/NADH ratio (Table 1). The maximal activities (V_{max}) of ADH in fresh liver homogenate-supernatants from fed rats were measured with 2.4 mM NAD^+ and the optimal ethanol concentration of 10 mM (Table 2). Based on these values for V_{max} and the substrate and product concentrations shown in Table 1, ADH activity was calculated from the steady-state rate equation to be 1.93 mmol per hour per animal (Table 2). The close agreement between this calculated rate and ethanol elimination rate measured *in vivo* [19] (Table 2) supports the contention that the alcohol oxidation rate can be predicted from the steady-state kinetic equation for purified ADH.

The K_m for NAD^+, 33 μM, is lower than the free NAD^+ concentration *in situ*, 0.5 mM; therefore, ADH is 93 percent saturated with respect to NAD^+. Since K_{iq} for NADH, 0.9 μM, is within the estimated range of concentration of this substrate *in situ* (Table 1), product inhibition by NADH must be a rate-limiting factor for ethanol oxidation by ADH. As

TABLE 1

CONCENTRATION OF NADH, ETHANOL AND ACETALDEHYDE IN FREEZE-CLAMPED RAT LIVER ONE HOUR AFTER INJECTION OF ETHANOL (2.0 g/kg)

NAD^+/NADH Ratio*	327 ± 27
Free NADH, μM†	1.6
Ethanol, mM	35.2 ± 4.4
Acetaldehyde, μM	9.5 ± 3.4

*Calculated from lactate/pyruvate ratios in freeze-clamped liver [20].

†Calculated from NAD^+/NADH ratio assuming that cytosolic free NAD^+ is 0.5 mM [20].

TABLE 2

COMPARISON OF CALCULATED ADH ACTIVITY WITH MAXIMAL ADH ACTIVITY AND ALCOHOL ELIMINATION RATES

	Rate mMol·hr^{-1}/rat
Maximal ADH Activity*	2.84
Calculated ADH Activity†	1.93
Alcohol Elimination Rate‡	1.92

*Liver cytosol activity measured in 0.5 M Tris-Cl, 2.8 mM NAD^+, 10 mM ethanol, pH 7.3 at 37° [20].

†Calculated from the rate equation, using substrate and product concentrations in Table 1.

‡Measured in fed rats after injection of 2 g ethanol/kg [19].

TABLE 3

EFFECT OF NADH AND ETHANOL CONCENTRATION ON ACTIVITY OF ADH CALCULATED FROM THE STEADY-STATE RATE EQUATION

	Cytosolic NADH	
	2 μM	0
Ethanol mM	v/V_{max} Percent	
10	69	80
40	66	75
100	53	61

Acetaldehyde and NAD^+ concentrations of 10 μM and 0.5 mM, respectively, were used to calculate v/V_{max} ratios from Equation 1.

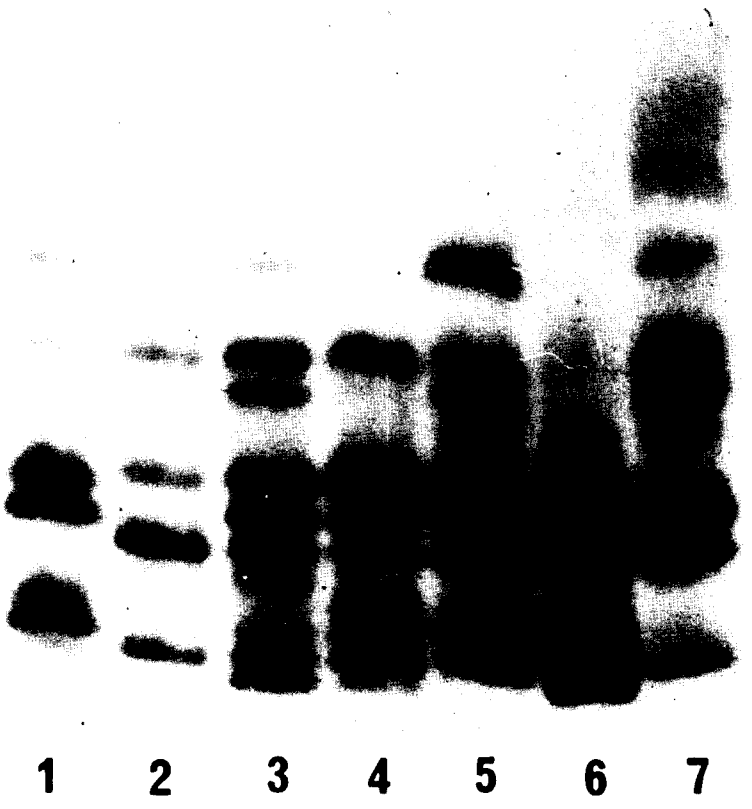

FIG. 1. Starch gel electrophoresis patterns of human liver ADH. Homogenate-supernatants of seven livers were applied to starch gels, electrophoresis was performed as described previously [3] and the gels were stained for ADH activity. The ADH phenotypes are named for the subunits observed. Thus, for isoenzymes with subunits produced at ADH_2 the phenotype is ADH_2 1-1 when only β_1 is observed and ADH_2 1-Ind when both β_1 and β_{Ind} subunits are observed. The enzyme phenotypes at ADH_3 are named similarly. The phenotypes are: 1=ADH_2 1-1, ADH_3 1-1; 2=ADH_2 Ind-Ind, ADH_3 1-1; 3=ADH_2 1-Ind, ADH_3 2-1; 4=ADH_2 1-Ind, ADH_3 1-1; 5=ADH_2 1-1, ADH_3 2-1; 6="Atypical" phenotype (4,24); 7=ADH_2 1-1, ADH_3 2-2.

calculated from Equation 1, activity is 69 percent of V_{max} at 2 μM NADH, 0.5 mM NAD^+, 10 μM acetaldehyde and 10 mM ethanol (Table 3). This ratio increases to 80 percent if it is assumed that NADH is very low. The 20 percent limitation below V_{max} in the absence of NADH is due to four approximately equal limitations; partial saturation by NAD^+, partial saturation by ethanol, substrate inhibition by ethanol and product inhibition by acetaldehyde. As shown in Table 3, the maximum calculated increase in v/V_{max} that can be obtained by assuming that NADH concentration is vanishingly small is 16 percent at the 3 different ethanol concentrations. This represents the greatest predicted degree of stimulation of ADH activity that can arise from the addition of agents that increase NAD^+/NADH. This calculated increase agrees reasonably well with the 15 to 30 percent increase in ethanol oxidation reported with rat hepatocytes after the addition of fructose [12] or *in vivo* after the addition of 2,4-dinitrophenol [15].

We also predict from Equation 1 that substrate inhibition by ethanol is a rate-limiting factor for ADH at alcohol concentrations above 10 mM. As shown in Table 3, when ethanol concentration is increased from 10 to 40 and 100 mM, the v/V_{max} ratio calculated with 2 μM NADH decreases from 69 to 66 and 53 percent, respectively. In support of this calculation, Braggins and Crow [6] recently reported that ethanol elimination rates measured *in vivo* in rats are lower at 130 mM ethanol than rates at 70 mM ethanol. This important limitation of enzyme rate at high ethanol concentration was reported some time ago by Dalziel and Dickinson [13] for horse liver ADH but it had not been fully appreciated in studies correlating rat liver ADH activity with ethanol elimination rates [9,20]. From these data, we conclude that the activity of rat liver ADH is limited principally by NADH product inhibition at low ethanol concentrations and both by NADH product inhibition and by ethanol substrate inhibition at high alcohol concentrations.

STEADY-STATE KINETICS OF HUMAN LIVER ALCOHOL DEHYDROGENASE ISOENZYMES AND THEIR ROLE IN REGULATING ALCOHOL METABOLISM IN MAN

The number of enzyme forms in human liver and the complexity of electrophoretic patterns on starch gels, as

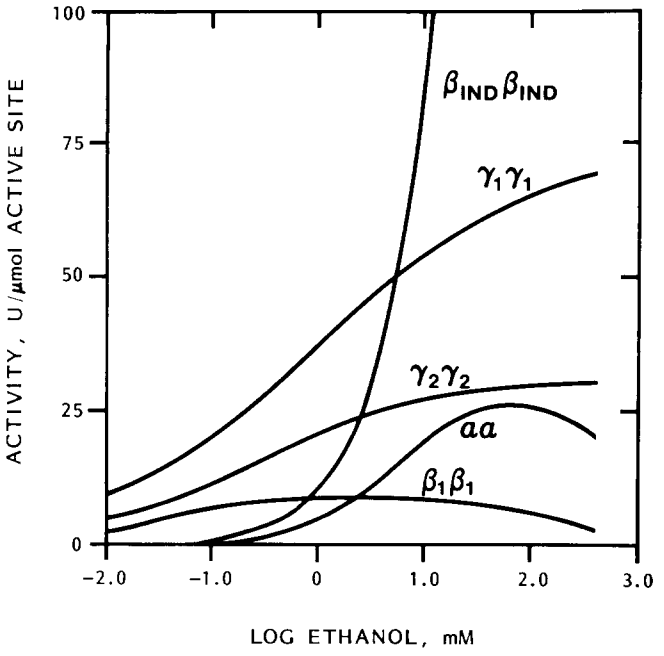

FIG. 2. Substrate saturation curves for homodimeric ADH isoenzymes. The substrate saturation curves were computed from the constants in Table 4 and Equation 3 ($\alpha\alpha$, $\beta_1\beta_1$ and $\beta_{Ind}\beta_{Ind}$) or Equation 2 ($\gamma_1\gamma_1$ and $\gamma_2\gamma_2$).

shown in Fig. 1, far exceeds that in the rat or any other known animal species. As many as 16 electrophoretically distinct enzyme forms have been identified in a single liver specimen [4,24]. Some years ago, a genetic model was proposed to account for this multiplicity of enzyme forms; 3 gene loci, ADH_1, ADH_2 and ADH_3, were proposed with two of their loci being polymorphic, ADH_2 and ADH_3 [24]. The starch gel electrophoresis patterns for seven different liver specimens and the proposed ADH phenotypes are shown in Fig. 1.

An efficient procedure for the purification of all ADH isoenzymes in human liver by affinity and ion-exchange chromatography was developed recently ([5,17], Bosron *et al.*, submitted for publication). We have isolated five homodimeric isoenzyme forms and π-ADH by this procedure and have determined their steady-state kinetic properties [5]. The five homodimers are $\alpha\alpha$, $\beta_1\beta_1$, $\beta_{Ind}\beta_{Ind}$, $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$, according to the nomenclature of Smith *et al.* [24] and Bosron and Li [4]. It has been proposed that the subunits in $\alpha\alpha$ are coded by ADH_1, those in $\beta_1\beta_1$ and $\beta_{Ind}\beta_{Ind}$ by the polymorphic ADH_2 locus and those in $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ by ADH_3.

The pH-activity profiles for $\alpha\alpha$, $\beta_1\beta_1$, $\gamma_1\gamma_1$, $\gamma_2\gamma_2$ and π-ADH have been determined with 33 mM ethanol and 2.4 mM NAD^+ and they all exhibit an optimum at 10.5 [3]. The pH-activity profile for $\beta_{Ind}\beta_{Ind}$ differs from the other forms; it has an optimum at 7.0 [3]. Ethanol saturation curves for the 5 homodimers were determined at pH 7.5, 25°, in 0.1 M NaPi with 2.4 mM NAD^+ as shown in Fig. 2. The kinetics of

TABLE 4

KINETIC CONSTANTS FOR ETHANOL OXIDATION BY HUMAN LIVER ADH ISOENZYMES AT pH 7.5

Isoenzyme	Hill Coefficient (h)	K_m or $S_{0.5}$ for Ethanol mM	V_{max} U/μmol active site*
π-ADH	1.01	34	20
$\alpha\alpha$	0.98	4.2	27
$\gamma_1\gamma_1$	0.50	1.00	81
$\gamma_2\gamma_2$	0.56	0.60	32
$\beta_1\beta_1$	0.93	0.048	9
$\beta_{Ind}\beta_{Ind}$	1.0	64	560

*Concentration of ADH active sites was determined by fluorescence titration of enzyme and isobutyramide with NADH [23].

π-ADH have been reported [2]. The kinetic data for all six forms were fit to the Hill equation [25]:

$$\log \frac{v}{V_{max} - v} = h(\log [S] - \log [S]_{0.5}) \qquad (2)$$

and to a form of the Michaelis-Menten equation derived for substrate inhibition [8].

$$\frac{v}{V_{max}} = \frac{S}{K_m + S + S^2/K_i} \qquad (3)$$

$\alpha\alpha$, $\beta_1\beta_1$, $\beta_{Ind}\beta_{Ind}$ and π-ADH all exhibited Hill coefficients of about 1.0 (Table 4) and the data fit Equation 3. Their V_{max} and K_m values are shown in Table 4; $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ had Hill coefficients of 0.50 and 0.56, respectively (Table 4), indicating negative cooperativity for substrate saturation [25]. The V_{max} values for $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$ were estimated from the fit of data at high ethanol concentration to the Michaelis-Menten equation [8] and $(S)_{0.5}$ values were calculated from the fit of data to Equation 2 [25]. The K_m or $(S)_{0.5}$ values for the 6 molecular forms in Table 4 varied over an extremely wide range of 1300-fold with that for $\beta_1\beta_1$ being the lowest and $\beta_{Ind}\beta_{Ind}$ being the highest. V_{max} values varied more than 60-fold with that for $\beta_{Ind}\beta_{Ind}$ being the highest and $\beta_1\beta_1$ being the lowest. Clearly, the kinetics of ethanol oxidation for the 2 alloenzymes produced at ADH_2, $\beta_1\beta_1$ and $\beta_{Ind}\beta_{Ind}$, differ to a greater degree than the 2 alloenzymes produced at ADH_3, $\gamma_1\gamma_1$ and $\gamma_2\gamma_2$.

Because of such large differences in the kinetic properties of the various isoenzymes of human liver ADH, correlation of ethanol elimination rates with the steady-state kinetic properties of the enzyme(s) would require *a priori*, knowledge of the isoenzyme composition or phenotype of the test subjects. Furthermore, it appears highly unlikely that the pharmacokinetics of ethanol elimination in man over a wide concentration range will fit either pseudo-zero ordered or simple Michaelis-Menten kinetics. Comparison of the ethanol elimination profiles of individuals with ADH_2 Ind-Ind or ADH_2 1-1 phenotypes should provide clarification of these issues.

REFERENCES

1. Arslanian, M. J., E. Pascoe and J. G. Reinhold. Rat liver alcohol dehydrogenase, purification and properties. *Biochem J* **125:** 1039–1047, 1971.
2. Bosron, W. F., T.-K. Li, W. P. Dafeldecker and B. L. Vallee. Human liver π-alcohol dehydrogenase: kinetic and molecular properties. *Biochemistry* **18:** 1101–1105, 1979.
3. Bosron, W. F., T.-K. Li and B. L. Vallee. New molecular forms of human liver alcohol dehydrogenase: isolation and characterization of $ADH_{Indianapolis}$. *Proc Natl Acad Sci USA* **77:** 5784–5788, 1980.
4. Bosron, W. F. and T.-K. Li. Genetic determinants of alcohol and aldehyde dehydrogenases and alcohol metabolism. *Semin Liver Dis* **1:** 179–188, 1981.
5. Bosron, W. F. and T.-K. Li. Kinetics of ethanol oxidation of four homodimeric human liver alcohol dehydrogenase isoenzymes. *Fed Proc* **41:** 921, 1982.
6. Braggins, T. J. and K. E. Crow. The effects of high ethanol doses on rates of ethanol oxidation in rats. *Eur J Biochem* **119:** 633–640, 1981.
7. Bücher, T., B. Brauser, D. Conze, F. Klein, O. Langguth and H. Sies. State of oxidation-reduction and state of binding in the cytosolic NADH-system as disclosed by equilibrium intracellular lactate/pyruvate in hemoglobin-free perfused rat liver. *Eur J Biochem* **27:** 301–317, 1972.
8. Cleland, W. W. Statistical analysis of enzyme kinetic data. *Methods Enzymol* **63A:** 103–138, 1979.
9. Cornell, N. W., K. E. Crow, M. Leadbetter and R. L. Veech. Rate-determining factors for ethanol oxidation *in vivo* and in isolated hepatocytes. In: *Alcohol and Nutrition,* Research Monograph No. 2, edited by T.-K. Li, S. Schenker and L. Lumeng. Washington, DC: Department of Health, Education and Welfare, 1979, pp. 315–330.
10. Crabb, D. W., T. A. Housinger, W. F. Bosron and T.-K. Li. Purification of multiple forms of rat liver alcohol dehydrogenase. *Alcoholism: Clin Exp Res* **5:** 146, 1981.
11. Crabb, D. W., W. F. Bosron and T.-K. Li. Kinetic characterization of rat liver alcohol dehydrogenase. *Fed Proc* **41:** 921, 1982.
12. Crow, K. E., K. M. Newland and R. D. Batt. Factors influencing rates of ethanol oxidation in isolated rat hepatocytes. *Alcoholism: Clin Exp Res* **6:** 293, 1982.
13. Dalziel, K. and F. M. Dickinson. The kinetics and mechanism of liver alcohol dehydrogenase with primary and secondary alcohols as substrates. *Biochem J* **100:** 34–46, 1966.
14. Hanozet, G. M., M. Simonetta, D. Barisio and A. Guerritore. Kinetic changes following modification of rat liver alcohol dehydrogenase by deoxycholate. *Arch Biochem Biophys* **196:** 46–53, 1979.
15. Israel, Y., J. M. Khanna and R. Lin. Effect of 2,4-dinitrophenol on the rate of ethanol elimination in the rat *in vivo*. *Biochem J* **120:** 447–448, 1970.
16. Kopun, M. and P. Propping. The kinetics of ethanol absorption and elimination in twins and supplementary repetitive experiments in singleton subjects. *Eur J Clin Pharmacol* **11:** 337–344, 1977.
17. Lange, L. G. and B. L. Vallee. Double-ternary complex affinity chromatography: preparation of alcohol dehydrogenases. *Biochemistry* **15:** 4681–4686, 1976.
18. Li, T.-K. Enzymology of human alcohol metabolism. *Adv Enzymol* **45:** 427–483, 1977.
19. Lumeng, L., W. F. Bosron and T.-K. Li. Quantitative correlation of ethanol elimination rates *in vivo* with liver alcohol dehydrogenase activity in fed, fasted and food-restricted rats. *Biochem Pharmacol* **28:** 1547–1551, 1979.
20. Lumeng, L., W. F. Bosron and T.-K. Li. Rate-determining factors for ethanol metabolism *in vivo* during fasting. In: *Alcohol and Aldehyde Metabolizing Systems*-IV, edited by R. G. Thurman. New York: Plenum Publishing Corp., 1980, pp. 489–496.
21. Markovic, O., H. Theorell and S. Rao. Rat liver alcohol dehydrogenase, purification and properties. *Acta Chem Scand* **25:** 195–205, 1971.
22. Pietruszko, R. Mammalian liver alcohol dehydrogenases. In: *Biochemical Pharmacology of Ethanol,* edited by E. Majchrowicz. New York: Plenum Press, 1975, pp. 1–31.
23. Plapp, B. V. Rate-limiting steps in ethanol metabolism and approaches to changing these rates biochemically. *Adv Exp Med Biol* **56:** 77–109, 1975.
24. Smith, M., D. A. Hopkinson and H. Harris. *Ann Hum Genet* **34:** 251–271, 1971.
25. Whitehead, E. P. Co-operativity and the methods of plotting binding and steady-state kinetic data. *Biochem J* **171:** 501–504, 1978.
26. Wratten, C. C. and W. W. Cleland. Product inhibition studies on yeast and liver alcohol dehydrogenase. Biochemistry **2:** 935–941, 1963.