Primary Deuterium and Tritium Isotope Effects upon V/K in the Liver Alcohol Dehydrogenase Reaction with Ethanol†

S. E. Damgaard

ABSTRACT: The primary isotope effect upon V/K when ethanol stereospecifically labeled with deuterium or tritium is oxidized by liver alcohol dehydrogenase has been measured between pH 6 and 9. The deuterium isotope effect was obtained with high reproducibility by the use of two different radioactive tracers, viz. ^{14}C and ^{3}H, to follow the rate of acetaldehyde formation from deuterium-labeled ethanol and normal ethanol, respectively. Synthesis of the necessary labeled compounds is described in this and earlier work referred to. V/K isotope effects for both tritium and deuterium have been measured with three different coenzymes, NAD^+, thio-NAD^+, and acetyl-NAD^+. With NAD^+ at pH 7, $^{D}(V/K)$ was 3.0 and $^{T}(V/K)$ was 6.5. With increasing pH, these values decreased to 1.5 and 2.5 at pH 9. The intrinsic isotope effect evaluated by the method of Northrop [Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–152, University Park Press, Baltimore] varies little with pH. It amounts to about 10 with NAD^+ and about 5 with the coenzyme analogues. Commitment functions and their dependence upon pH calculated in this connection appear to be in agreement with known kinetic parameters of liver alcohol dehydrogenase. This assay method was also applied in vivo in the rat. Being a noninvasive method because only trace amounts of isotopes are needed, it may yield information about alternative routes of ethanol oxidation in vivo. In naive rats at low concentrations of ethanol, it confirms the discrete role of the non alcohol dehydrogenase systems.

In the study of the mechanism of liver alcohol dehydrogenase, valuable information has been obtained by measurement of kinetic isotope effects, viz. the factor by which the normal rate of reaction is decreased by isotopic substitution into one reactant. When substitution with ^{2}H or ^{3}H into ethanol comprises position *pro*-1*R*, it results in a primary isotope effect on the hydrogen transfer[1] step in the mechanism, and it is generally believed that as a reasonable approximation this so-called intrinsic isotope effect alone is responsible for the observed kinetic isotope effects upon V and V/K (Cleland, 1975). In the following, these two primary isotope effects will be termed $^{D}(V)$ and $^{D}(V/K)$ for (1*R*)-[^{2}H]ethanol and $^{T}(V/K)$ for (1*R*)-[^{3}H]ethanol. It follows that the isotope effects upon V and V/K cannot exceed the value of the intrinsic isotope effect, and they will usually be smaller than this, viz. when other steps in the mechanism contribute to the limitation of the net catalytic rate of the reaction (klinman, 1978). $^{D}(V)$ is about 1.3 (Baker, 1962; Bush et al., 1973; Plapp et al., 1973; Dworschack & Plapp, 1977). A value of about 2 for $^{D}(V/K)$ has been obtained by Baker (1962),[2] and a value of 4.1 for $^{T}(V/K)$ was obtained in the presence of semicarbazide by Palm et al. (1968). Estimates of the intrinsic deuterium isotope effect of about 5–6 have been obtained with transient stopped-flow methods (Shore & Gutfreund, 1970; Brooks & Shore, 1971; Brooks et al., 1972). $^{D}(V)$ values up to 5.2 with [U-^{2}H]ethanol were obtained with preparations of chemically modified enzyme which apparently conforms to a mechanism in which the net catalytic rate is limited by hydrogen transfer to a significantly higher extent than in the native enzyme (Plapp et al., 1973; Dworschack & Plapp, 1977). With the native enzyme circumvention of the rate-limiting step in the mechanism, the one at which NADH dissociates from the apoenzyme allows the intrinsic isotope effect to be expressed more markedly in $^{D}(V)$. When ethanol oxidation was coupled with lactaldehyde reduction, NADH dissociation from the apoenzyme was circumvented, and a value of 4.2 was obtained for $^{D}(V)$ (Gershman & Abeles, 1973). When NAD^+ was substituted with thio-NAD^+, the reduced form of which dissociates more rapidly than NADH from the apoenzyme, Baici et al. (1975) were able to obtain $^{D}(V)$ values of 2–2.5. The interpretations of the results mentioned above are corroborated by the fact that $^{D}(V)$ values found with alcohols for which the hydrogen transfer proceeds more slowly than with ethanol are significantly higher than those obtained with ethanol (Brooks & Shore, 1971; Gershman & Abeles, 1973). The intrinsic hydrogen isotope effect in the alcohol dehydrogenase reaction appears to be significantly higher than the kinetic isotope effects upon V and V/K obtainable by conventional enzyme kinetic methods. Its actual magnitude remains, however, to be established. According to Northrop (1975, 1977), the intrinsic isotope effect of an enzymatic reaction can be estimated from values of $^{D}(V/K)$ and $^{T}(V/K)$ obtained under identical conditions provided that the isotope effect upon the equilibrium of the reaction is small. For the alcohol dehydrogenase reaction, the equilibrium effect with ^{2}H is close to 1.07 (Cook et al., 1980), small enough to permit the use of this method. In the present work, the intrinsic isotope effects on ethanol oxidation with NAD^+, acetyl-NAD^+, and thio-NAD^+ in the liver alcohol dehydrogenase reaction have been determined at various values of pH between 6 and 9 by the method described by Northrop (1975, 1977). The values for the intrinsic isotope effect and the commitment function[3] obtained in this study are discussed in relation to the catalytic mechanism. In order to measure $^{D}(V/K)$ as accurately as possible under conditions where dissociation of reduced co-

† From the Department of Biochemistry A, The Panum Institute, University of Copenhagen, DK-2200 Copenhagen, Denmark. *Received March 18, 1981.* This investigation was supported by F. L. Smidth and Co. A/S' Jubilaeumsfond.

[1] The term hydrogen transfer is chosen because it has not been demonstrated whether the transferred entity is a hydride ion or a hydrogen radical (Klinman, 1978).

[2] In the work referred to, the nomenclature of Dalziel (1957) was used. Therefore V/K is designated $1/\phi_2'$.

[3] The commitment functions are algebraic combinations of rate constants and substrate concentrations. They appear in the expression for $^{D}(V/K)$. Their magnitudes determine to what extent the intrinsic isotope effect is expressed in $^{D}(V/K)$ [cf. Northrop (1977)].

0006-2960/81/0420-5662$01.25/0

enzyme has no influence, a radioactive method used earlier with catalase (Damgaard, 1980a) was employed. A significant difference from earlier studies is the use of stereospecifically labeled substrate which minimizes secondary isotope effects. This method has also been used in two in vivo experiments where significant $^{D}(V/K)$ values were obtained with ethanol.

Experimental Procedures

Chemicals were of analytical grade when not otherwise specified. Acetaldehyde was twice distilled from water. Semicarbazide hydrochloride was from Hopkin & Williams (Chadwell Heath, Essex, U.K.). [U-^{2}H]Ethanol (99% pure, Uvasol grade) was from Merck (Darmstadt, Germany). [1,1-$^3H_2$]Ethanol was from ICN, Irvine, CA. [1-^{14}C]Ethanol (57 Ci/mol), D-[6-^{3}H]glucose (500 Ci/mol), and D-[1-^{3}H]-galactose (5.2 Ci/mol) were purchased from Amersham (Bucks, U.K.). [1,2-$^{14}C_2$]Acetaldehyde (8 Ci/mol) as paraldehyde was from New England Nuclear.

Horse liver and yeast alcohol dehydrogenase, rabbit muscle lactate dehydrogenase, ox liver catalase, pig heart diaphorase, grade I, *Candida bodinii* alcohol oxidase, yeast acetyl-CoA synthetase, pig heart citrate synthetase, pig heart malate dehydrogenase, *Pseudomonas fluorescens* β-galactose dehydrogenase, and 3-acetylpyridine adenine dinucleotide were from Boehringer (Mannheim, Germany). Thio-NAD^+ was from Sigma. The NAD^+ coenzyme analogues were checked according to Siegel et al. (1959) and Stein et al. (1963).

[2-^{3}H]Ethanol was made by fermentation of D-[6-^{3}H]-glucose with bakers' yeast and purified and tested as described by Damgaard (1980a). (1*R*)-[1,2-$^{14}C_2$,1-^{2}H]Ethanol was synthetized enzymatically from [1,2-$^{14}C_2$]acetaldehyde and L-[2-^{2}H]lactate as described by Damgaard (1980a). [1-^{14}C]Acetaldehyde was made from [1-^{14}C]ethanol by enzymatic oxidation. To 0.5 mCi of [1-^{14}C]ethanol (8.8 μmol) in 1.0 mL of water was added 1 mg of alcohol oxidase and 2 mg of catalase. This mixture was kept with slow stirring in a 10-mL glass tube with an oxygen atmosphere at room temperature for 24 h. Then 20 mg of semicarbazide hydrochloride was added followed by 40 mg of K_2HPO_4 and 0.5 mL of 96% ethanol 1 h later. This mixture was evaporated to dryness in a stream of nitrogen, and in three steps, 2 × 2 mL of 95% ethanol and 1 × 1.0 mL of [U-^{2}H]ethanol was added and evaporated. Finally, the dry matter was dissolved in 6 mL of 0.6 M H_2SO_4, and the acetaldehyde thus liberated was collected in ice-cold water as described for [1-^{3}H]acetaldehyde (Damgaard, 1977). The yield on the basis of [1-^{14}C]ethanol was 68–79%. (1*R*)-[1-^{14}C,1-^{2}H]Ethanol was synthetized by letting [1-^{14}C]acetaldehyde (2 μmol) diffuse at 22 °C from a centerwell in a closed 15-mL conical glass stoppered flask into 250 μL of 0.01 potassium phosphate, pH 6.5, containing 100 μg of dialyzed yeast alcohol dehydrogenase, 6 mg of [U-^{2}H]ethanol (116 μmol), and 25 μg NAD^+ contained in the main compartment of the same flask placed in an oblique position. After 24 h, all water, ethanol, and acetaldehyde were collected in the centerwell by condensing all vapor on a cold finger (containing solid CO_2) over this centerwell. The contents of the centerwell was then mixed with semicarbazide hydrochloride to 15 mM and K_3PO_4 to bring the pH to 6–7. After this, ethanol and water were isolated from the acetaldehyde semicarbazone by renewed destillation onto a cold finger in an analogous setup. The yield in ethanol on the basis of [1-^{14}C]acetaldehyde was about 80%. The diffusion of [1-^{14}C]acetaldehyde into the reaction mixture ensures a sufficiently slow reduction of this compound to keep the steady-state concentration of NAD^+ low enough to prevent significant oxidation of [1-^{14}C]acetaldehyde to [1-^{14}C]acetate by the aldehyde dehydrogenase activity found to be present in the yeast alcohol dehydrogenase preparation and every other commercial alcohol dehydrogenase preparation tested.

This condensate was then diluted with nonisotope ethanol and [2-^{3}H]ethanol to contain less than 5% deuterium in the ethanol. In experiments with 10% deuterium in the ethanol, the $^{D}(V/K)$ isotope effect obtained was unchanged. The specific radioactivities of ^{14}C and ^{3}H in the ethanol substrate mixture was 22–31 and 80–147 cpm/nmol of ethanol, respectively. (1*R*)-[1-^{3}H]Ethanol was prepared enzymatically from D-[1-^{3}H]galactose as described earlier (Damgaard, 1977).

The sodium salt of (4*R*)-[4-^{3}H]NADH was prepared as described by Rafter & Colowick (1957) by using [1,1-$^3H_2$]-ethanol. A 73-mg sample of (4*R*)-[4-^{3}H]NADH was converted to [4-^{3}H]NAD^+ in 6 mL of 50 mM Tris-HCl, pH 7.5, by adding 0.2 mL of a 3 mM methylene blue solution in ethanol and 0.5 mg of diaphorase. The reaction mixture was stirred in an oxygen atmosphere and the disappearance of NADH followed by monitoring A_{340} in small samples. After 108 min, the reaction was complete, and oxygen was substituted with nitrogen. Then 400 μL of formic acid and 31 mL of ice-cold acetone was added to precipitate NAD^+. NAD^+ was redissolved in water and reprecipitated in the same manner. This precipitate was then dried with acetone + ether (1:1 v/v) and kept in a desiccator over $CaCl_2$. The [4-^{3}H]-NAD^+ was found to be "enzymatically active" with an equivalent weight of 800. It contained traces of methylene blue. The yield was 40 mg, ~54%.

Enzyme Purifications. Liver alcohol dehydrogenase was purified from 190 g of female Wistar rats essentially according to Reynier (1969) with the improvements of Markovic & Theorell (1972) in the presence of 5% ethanol. The specific catalytic activity of the final product was 1.1 μmol min^{-1} (mg of protein)$^{-1}$. No separation of isoenzymes was attempted.

Liver alcohol dehydrogenase was purified from 24–36 kg of female pigs of Danish landrace, employing the prescriptions for preparation of the human liver enzyme of Blair & Vallee (1966). The specific catalytic activity obtained in the best fraction was 6 μmol min^{-1} (mg of protein)$^{-1}$, corresponding to 18 times purification. The overall yield was 3%. Alcohol dehydrogenase activity was measured in the direction of acetaldehyde reduction according to Büttner (1965).

A crude preparation of mitochondrial adehyde dehydrogenase from rat liver was made according to Tottmar (personal communications). Mitochondria were prepared from two 200-g female Wistar rats according to Grunnet (1970). The mitochondria were suspended in water (1 mL/g of liver) and frozen and thawed 3 times. After centrifugation, the supernatant was isolated, and its protein content adjusted to 1.0–1.2 mg/mL and precipitated with ammonium sulfate at 4 °C. The precipitate obtained between 40% and 60% saturation was dissolved in 5 mL of 10 mM potassium phosphate at pH 7.5. It contained most of the acetaldehyde dehydrogenase activity. The preparation was fairly stable at −70 °C. The best preparations contained 0.1 μmol min^{-1} mL^{-1} aldehyde dehydrogenase, about 0.015 μmol min^{-1} mL^{-1} alcohol dehydrogenase, and 10 mg of protein/mL. Aldehyde dehydrogenase activity was measured according to assay II by Grunnet (1973). Protein was determined according to Groves et al. (1968) or, when the concentration was large enough, according to Gornall et al. (1949).

Assay for V/K Isotope Effects. The ^{2}H or ^{3}H V/K isotope effect on the oxidation of ethanol was measured at 37 °C. Standard conditions were pH 7.0, 4–10 mM of ethanol, and either 0.5 mM of NAD^+ and 33 mM of semicarbazide or 6

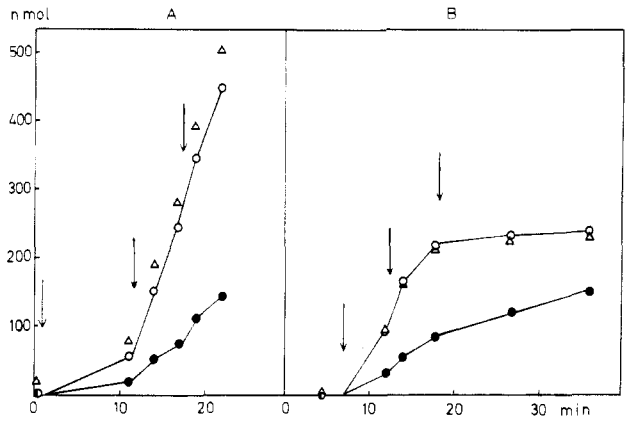

FIGURE 1: Oxidation of (1*R*)-[1-^{14}C,1-^{2}H]ethanol and [2-^{3}H]ethanol mixture at pH 7.0 and 37 °C. The symbol △ represents nmoles of NADH formed as calculated from the increase in absorption at 340 nm. The symbols ○ and ● represent nmoles of acetaldehyde as calculated from the increase in ^{3}H and ^{14}C radioactivity in the semicarbazone fraction, respectively. The arrows indicate addition of horse liver alcohol dehydrogenase, 10, 40, and 80 μg of protein. The first addition initiates the reaction. Assay conditions: (A) NAD^+, 0.5 mM; ethanol, 5.9 mM; semicarbazide, 33 mM. (B) NAD^+, 6.0 mM; ethanol, 5.9 mM; without semicarbazide. $^{D}(V/K)$: (A) 2.97, 2.92, 3.14, 3.01, and 3.08. (B) 3.01, 3.03, 2.62, 1.75, and 1.56.

mM of NAD^+ and no semicarbazide. For $^{D}(V/K)$ determination, (1*R*)-[1-^{14}C,1-^{2}H]ethanol or (1*R*)-[1,2-$^{14}C_2$,1-^{2}H]-ethanol was mixed with [2-^{3}H]ethanol and used. For $^{T}(V/K)$ determination, (1*R*)-[1-^{3}H]ethanol was used.

In the pH interval between 6.0 and 8.0, the buffer was 0.10 M potassium phosphate. Between pH 8.0 and 9.0, 0.05 M potassium pyrophosphate with 3.6 mM glycine was used. The reactions proceeded in 3.1 mL of buffered medium in spectrophotometer cells, light path 1.00 cm, with Teflon lids, and the reactions were followed by recording the change in absorption due to formation of reduced coenzyme. NADH and acetyl-NADH were followed continuously at 340 nm, while thio-NADH was measured at 2-min intervals for 8 s at 398 nm. The specific absorption coefficients used were 6.22, 6.67 (Siegel et al., 1959), and 11.9 mM cm^{-1} (Stein et al., 1963) for the three reduced coenzymes. These values agreed well with the radioactive ^{3}H determination of product formation in the $^{D}(V/K)$ assay (cf. *Figure* 1). The ranges of concentrations of substrates and enzymes used in the assays were ethanol, 1–86 mM, oxidized coenzyme, 0.1–10 mM, and horse liver alcohol dehydrogenase, 2–132 μg. During a single assay, the alcohol dehydrogenase concentration was usually changed by a factor of 5–10.

During the progress of the reaction, samples of 0.30 mL were removed and at once mixed with 4-methylpyrazole or $AgNO_3$ to a final concentration of 1 mM to stop the reaction. At high concentrations of ethanol, 10 mM 4-methylpyrazole was used. At the end of the experiment, one sample of the reaction mixture was deproteinized in ice-cold PCA. The clear neutralized supernatant was used to control the specific radioactivities of ^{3}H and ^{14}C in ethanol.

The samples were transferred to counting vials and evaporated to dryness to measure $^{D}(V/K)$. To samples without semicarbazide was added 40 μmol of this compound. Following addition of 0.3 mL of 96% ethanol and reevaporation to dryness 3 times, the dry matter was dissolved in 1.0 mL of water and counted for ^{3}H and ^{14}C radioactivities. Omission of semicarbazide resulted in the complete disappearance of all radioactivity from the samples. Calculation of $^{D}(V/K)$ from the obtained radioactivities and the specific radioactivities of ethanol is presented under Results (Figure 1).

To measure $^{T}(V/K)$, each sample was applied on a separate track of a sliced Whatman No. 1 paper (Type 3350, Frisenette, Ebeltoft, Denmark) and eluted overnight with 96% ethanol to remove all radioactivity in ethanol. After the paper was dried, the spots were cut out, and the paper was extracted in counting vials with 1.0 mL of water prior to counting of ^{3}H radioactivity. No ^{3}H radioactivity was found on the paper below the spot. All ^{3}H radioactivity in [4-^{3}H]NAD^+ added to the reaction mixture is an experiment with unlabeled ethanol and all (4*R*)-[4-^{3}H]NADH added to samples from a blind experiment were recovered in this procedure. $^{T}(V/K)$ was calculated for each sample as the ratio between the amount of the product formed as calculated from the increase in A_{340} or A_{398} relative to the amount of product calculated from the ^{3}H radioactivity recovered in the spot of the paper using the specific ^{3}H radioactivity in the ethanol substrate.

Measurement of $^{D}(V/K)$ in the in Vivo Rat. Two female Wistar rats of 195 and 200 g received by stomach tube 2.64 mmol of ethanol as a 1.55-mL solution in water. The ethanol contained (1*R*)-[1-^{14}C,1-^{2}H]ethanol as a trace, less than 5% deuterium. The specific radioactivity of total ethanol was measured to be 6.69 and 20.68 cpm/nmol in the two experiments.

Fifteen minutes later, the rats were decapitated. The blood was collected in two or three heparinized plastic tubes, and from these, 2 mL was precipitated with equal volumes of 1 M ice-cold PCA. On the neutralized supernatant, ethanol and acetate were determined by enzymatic analysis. From the remainder of this supernatant, acetate was isolated by diffusion according to Lundquist (1962) and freed from labeled ethanol by repeated evaporations of added nonradioactive ethanol. Then the acetate was redissolved in the original volume of water and its specific radioactivity determined by counting and enzymatic analysis.

Counting of Radioactivities. Aqueous samples (1.0 mL) in 10 mL of scintillation liquid prepared as described by Anderson & McClure (1973) were counted for ^{3}H or ^{3}H and ^{14}C simultaneously as described earlier (Damgaard, 1977, 1980a).

Enzymatic Analysis. The following assays were used: ethanol (Bonnichsen, 1962), acetaldehyde (Bergmeyer, 1962), and acetate (Bergmeyer & Möllering, 1970).

Results

Two $^{D}(V/K)$ isotope effect determination runs are shown in Figure 1. The ratio of product calculated from ^{3}H radioactivity to product calculated from ^{14}C radioactivity is the $^{D}(V/K)$ isotope effect. This is the case because all ^{14}C radioactivity in the substrate is associated with deuterium in position *pro*-1*R*, while ^{3}H in the methyl group is converted to product without isotope effect. No difference in $^{D}(V/K)$ was observed between substrates with ^{14}C labeled in position 1 alone compared to those with label in both carbon atoms. In experiment A, the presence of semicarbazide ensures irreversibility by keeping the concentration of free acetaldehyde low. As a consequence, $^{D}(V/K)$ remains constant throughout the assay while the enzyme concentration is increased 8 times. In experiment B, semicarbazide is absent, and in parallel with product accumulation, the $^{D}(V/K)$ isotope effect decreases. Initial $^{D}(V/K)$ values are, however, identical with the values obtained in experiment A, which justifies the use of semicarbazide. The observed decrease in $^{D}(V/K)$ in experiment B can be attributed to formation of [1-^{14}C]ethanol from [1-^{14}C]acetaldehyde. When [1-^{14}C]ethanol was added to the substrate mixture to represent 10% or 40% of the total ^{14}C radioactivity, isotope effects of 2.52 ± 0.12 (4) and 1.67 ±

Table I: Primary ^2H Isotope Effect in EtOH Oxidation by Alcohol Dehydrogenase (pH 7.0, 33 mM Semicarbazide, 37 °C)[a]

enzyme	EtOH (mM)	NAD^+ (mM)	isotope effect ± SD	n	in absence of semicarbazide: NAD^+ (mM)	in absence of semicarbazide: I ± SD
horse liver	4.4–5.9	0.5	3.00 ± 0.02	5	6.6	2.79 ± 0.10 (5)
horse liver	7.6–14.2	0.5	3.08 ± 0.08	5	6.6	3.00 ± 0.17 (4)
horse liver	19.8–21.6	0.5	3.08 ± 0.07	5	6.6	2.90 ± 0.10 (3)
horse liver	62.0–86.5	0.5	3.00 ± 0.19	5	6.6	2.81 ± 0.11 (5)
horse liver	9.9	6.4	3.00 ± 0.07	5		
rat liver	4.7–5.4	0.5	3.11 ± 0.10	5	6.7	2.65 ± 0.10 (5)
rat liver	7.0–8.6	0.5	3.12 ± 0.09	5	6.6	2.86 ± 0.05 (5)
pig liver	4.0–4.9	0.5	2.87 ± 0.02	5	6.6	2.79 ± 0.12 (4)

[a] Variation of source of enzyme, concentration of ethanol, and NAD^+.

Table II: Determination of $^D(V/K)$ Isotope Effect upon Ethanol Oxidation in the in Vivo Rat[a]

rat no.	concn (mM) in blood at 15 min: ethanol	concn (mM) in blood at 15 min: acetate	sample no.	recovery (%) of acetate after diffusion	ethanol (cpm/μmol)	acetate (cpm/μmol)	$^D(V/K)$ isotope effect
1	3.5	0.95	1	97	20684	8759	2.36
			2	100		9136	2.26
2	1.6	0.84	1	92	6689	2322	2.88
			2	83		2162	3.09
			3	80		2675	2.50

[a] The labeled ethanol was given by stomach tube 15 min before sacrifice, and acetate was sampled, isolated, and analyzed as described under Experimental Procedures.

0.03 (5), respectively, were obtained in the presence of semicarbazide. These results conform to

$$T = \frac{\theta(1 - F)}{1 - \theta F}$$

where F, $0 \leq F < 1$, is the fraction of ^{14}C not associated with deuterium and T is the true and θ is the observed $^D(V/K)$ isotope effect. In experiments with all ^{14}C radioactivity present as [1-^{14}C]ethanol ($F = 1$), the isotope effects measured were 1.03 ± 0.10 (5) and 1.00 ± 0.06 (5) without and with semicarbazide, respectively. As an alternative to semicarbazide, addition of pyruvate to 1.0 mM and 100 μg of lactate dehydrogenase in the standard assay prevented significant reversibility by keeping NADH below 1.6 μM as judged from A_{340}. Despite an acetaldehyde accumulation to about 0.7 mM (2 μmol) over 20 min, the obtained $^D(V/K)$ was 2.98 ± 0.10 (5). This experiment confirmed that the decrease in $^D(V/K)$ in experiment B of Figure 1 was not due to acetaldehyde per se but to actual reversibility. In other experiments without semicarbazide, it was attempted to restrict sampling to the initial phase of the reaction.

Table I shows experiments with different liver alcohol dehydrogenases as well as different concentrations of ethanol and NAD^+. It may be concluded that changes in the concentration of ethanol between 4 and 86 mM have no influence on the magnitude of the isotope effect. Likewise various liver alcohol dehydrogenases differ little with respect to $^D(V/K)$. To these results may be added that the presence of 10 μM pyrazole while the specific catalytic activity of alcohol dehydrogenase is decreased to about 10% did not influence the $^D(V/K)$ isotope effect obtained, 2.99 ± 0.11 (7).

Figure 2 presents the results of measurements of $^D(V/K)$ at various pH values between 6 and 9. It appears that the pH sensitivity of $^D(V/K)$ depends upon the coenzyme used in the reaction. With NAD^+, the natural coenzyme, the titration curve ($pK \sim 8.4$) lies below that with thio-NAD^+ ($pK \geq 8.6$) but above that with acetyl-NAD^+ ($pK \sim 8$). Table II contains the results from measurement of the $^D(V/K)$ on ethanol oxidation in the in vivo rat. As with the isolated enzymes, (1R)-[1-^{14}C,1-^{2}H]ethanol was mixed into the ethanol substrate. [2-^{3}H]Ethanol was not used in order to circumvent a possible

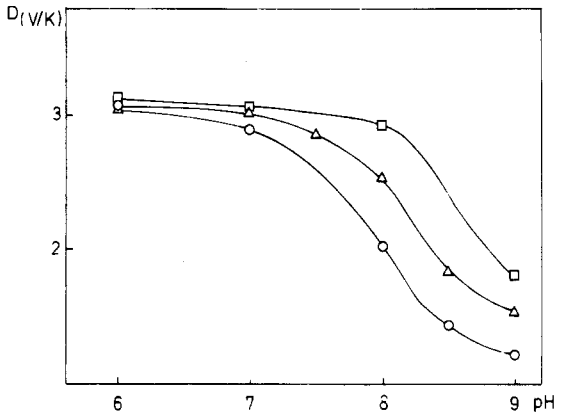

FIGURE 2: pH dependence of the first-order kinetic isotope effects with deuterium, $^D(V/K)$, upon oxidation of ethanol with NAD^+ (△), acetyl-NAD^+ (○), and thio-NAD^+ (□), all 0.5 mM in the horse liver alcohol dehydrogenase reaction. Values of standard deviation were too small to be presented graphically. Identical $^D(V/K)$ values were obtained at pH 8.0 with potassium phosphate and pyrophosphate buffers. Not less than four determinations are represented in each point. Assay conditions as under Experimental Procedures and Figure 1A.

detrition of the methyl group in vivo (Cronholm, 1979). Instead, acetate was isolated from the blood and its specific radioactivity determined. The factor by which this was decreased compared to the specific radioactivity of ethanol is the $^D(V/K)$ isotope effect. Acetate and acetaldehyde should have the same specific radioactivity since no discernible isotope effect upon oxidation of [1-^{14}C]acetaldehyde to [1-^{14}C]acetate is expected and formation of nonlabeled acetate from other sources is believed to be negligible.

The $^T(V/K)$ isotope effect was determined with (1R)-[1-^{3}H]ethanol at various pH values between 6 and 9 with different coenzymes under conditions similar to those used for the $^D(V/K)$ isotope effect determinations shown in Figure 2. The results are shown in Figure 3. That this assay method is less accurate than the radioactive one used with deuterium is apparent from the standard deviation values indicated on the graphs. The pK values appear to be in the same range as for $^D(V/K)$ (Figure 2). For $^T(V/K)$ pH dependence, an additional

Table III: Intrinsic Isotope Effects in the Liver Alcohol Dehydrogenase Reaction Calculated from the $^{D}(V/K)$ and $^{T}(V/K)$ Values Shown in Figures 2 and 3 according to the Method of Northrop (1977)[a]

coenzyme	isotope effect	pH 6.0	pH 7.0	pH 8.0	pH 9.0
NAD^+	^{D}k	4.6 (2.6–8.9)	8.3 (5.9–12.0)	11.6 (8.3–16.8)	9.9 (8.2–11.8)
	^{T}k	9.1 (3.9–23.2)	21.2 (12.9–36.0)	34.3 (21.2–58.5)	27.1 (21.0–35.1)
thio-NAD^+	^{D}k	2.5 (1.2–5.5)	4.4 (2.3–8.4)	3.9 (2.3–7.0)	4.8 (3.1–7.5)
	^{T}k	3.7 (1.2–11.6)	8.5 (3.4–21.5)	7.1 (3.2–16.4)	9.5 (5.2–18.1)
acetyl-NAD^+	^{D}k	3.6 (3.3–3.8)	5.7 (3.7–8.9)		5.3 (4.0–6.9)
	^{T}k	6.3 (5.6–7.0)	12.2 (6.7–23.3)		11.2 (7.3–16.0)

[a] The intervals in parentheses are the range for the intrinsic isotope effect calculated on the basis of $[^{D}(V/K) - 1]/[^{T}(V/K) - 1] \pm SD$.

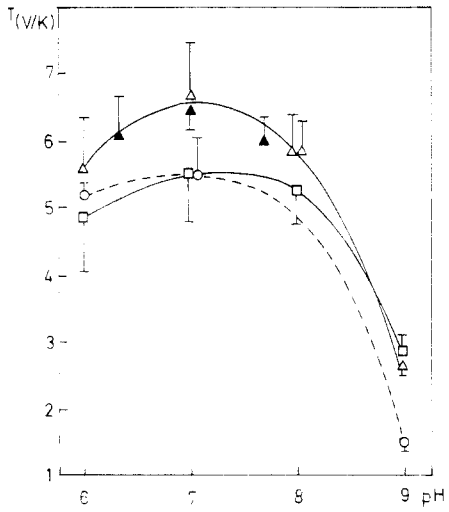

FIGURE 3: pH dependence of the first-order kinetic isotope effects with tritium, $^{T}(V/K)$, upon oxidation of ethanol with NAD^+ (△), acetyl-NAD^+ (○), and thio-NAD^+ (□), 0.6–1.0 mM in the horse liver alcohol dehydrogenase reaction. The vertical lines indicate values of standard deviate. Identical $^{T}(V/K)$ values were obtained at pH 8.0 with potassium phosphate and pyrophosphate buffers, two points shown on the graph. The symbol ▲ represents experiments with NAD^+ and aldehyde dehydrogenase instead of semicarbazide. Not less than four determinations are represented in each point. Assay conditions as described under Experimental Procedures.

proton base $pK \leq 6$ appears to be important. As an alternative to the use of semicarbazide, the reaction was rendered irreversible by addition of 13 milliunits of mitochondrial NAD dependent aldehyde dehydrogenase. With this enzyme instead of semicarbazide, the $^{T}(V/K)$ values were not different from those obtained with semicarbazide. The enzyme from pig and rat liver was also studied and did not differ from that of horse liver at pH 7.0. Addition of products in the absence of semicarbazide had different effects in respect to decreases in $^{T}(V/K)$. With added 7 and 40 μM acetaldehyde, $^{T}(V/K)$ initial values were 3.4 and 1.9, respectively. With 20 μM NADH, $^{T}(V/K)$ was 6.2–5.9. In the presence of both products, 20 μM NADH and 8 μM acetaldehyde, $^{T}(V/K)$ was decreased to 1.3–1.5. Thus an obvious difference in sensitivity to the first and second product exists. This is in agreement with the ordered bi-bi mechanism of this enzyme.

Table III contains the intrinsic isotope effects calculated by the method of Northrop (1977). To do this, the quotient between $^{D}(V/K) - 1$ and $^{T}(V/K) - 1$ is calculated. This quotient is taken to be equal to $^{D}k - 1$ divided by $^{D}k^{1.44} - 1$ where ^{D}k is the intrinsic deuterium isotope effect. The standard deviation of the V/K isotope effects is used to calculate the standard deviation of this quotient and thence the range for the intrinsic isotope effect corresponding to ± standard deviation. These ranges are shown in parentheses. The highest values for the intrinsic isotope effect are obtained with NAD^+. At pH 6, the intrinsic isotope effect appears lower than at pH 7–9, where similar values were obtained with each of the three coenzymes. The accuracy required in the determination of the kinetic isotope effects is quite high for this calculation. Although the SD is about 10%, for $^{T}(V/K)$ and much less for $^{D}(V/K)$, the ranges obtained for the intrinsic isotope effects are large, especially at low pH values.

Discussion

By the use of the doubly labeled ethanol substrate, viz. (1*R*)-[1-^{14}C,1-^{2}H]ethanol, ^{14}C serves as a tracer for molecules converted with an intrinsic deuterium isotope effect. Simultaneous use of [2-^{3}H]ethanol permits the tracing of molecules converted without isotope effects. In this way, the number of conversion steps involved in the expression for $^{D}(V/K)$ is brought to a minimum, because only steps between binding of labeled substrate and dissociation of ^{14}C-labeled acetaldehyde appears (Cleland, 1975; Northrop, 1975, 1977). The similarities found between the $^{D}(V/K)$ isotope effects for different species of alcohol dehydrogenase reveal that the central mechanism in this enzymatic reaction is more similar in these species than are reactions responsible for substrate specificity and molecular activities (Wratten & Cleland, 1965; Arslanian et al., 1971; Marcovič et al., 1971). Simplicity of the expression for the $^{D}(V/K)$ effect in combination with the use of stereospecific labeling with deuterium reduces the possibilities of interference from secondary isotope effects when the intrinsic isotope effect is evaluated later in this discussion by the method of Northrop (1977).

The exchange of the product acetaldehyde backward to ethanol in the alcohol dehydrogenase reaction does not change the ratio of ^{3}H to ^{14}C in acetaldehyde and does not then impair the method, provided the amount of [^{14}C]ethanol remains an insignificant part of the total ^{14}C radioactivity in ethanol. These conclusions are supported by the experiments shown in Figure 1 and Table I as well as the experiment in which semicarbazide was replaced by lactate dehydrogenase and pyruvate. In this experiment, acetaldehyde was allowed to accumulate. Furthermore, the experiments with the in vivo rat, Table II, demonstrate that the method is not impaired by the in vivo conversion of the acetaldehyde to acetate. A $^{D}(V/K)$ isotope effect almost as large as the one obtained in vitro with purified alcohol dehydrogenase was obtained with this method. Gershman & Abeles (1973) were unable to demonstrate a $^{D}(V/K)$ isotope effect on the same reaction in the in vivo mouse because their method was dependent upon the rate-limiting dissociation of NADH from the apoenzyme, which suppressed the isotope effect with labeled ethanol. With labeled 2-propanol, where hydrogen transfer is a rate-limiting reaction, they were able to demonstrate the isotope effect in vivo. The slightly lower $^{D}(V/K)$ isotope effect measured in the in vivo rat compared to in vitro need not reflect a significant in vivo exchange of ^{14}C acetaldehyde to ethanol but is likely to reflect the operation of non alcohol dehydrogenase pathways like catalase and MEOS in the oxidation of ethanol (Ugarte & Iturriaga, 1976). Thus for catalase-mediated oxidation of ethanol, a $^{D}(V/K)$ isotope effect of 1.9 has been reported

Damgaard, 1980a) and for MEOS an even lower one (Damgaard, 1980b). This view is supported by the finding of very low blood concentrations of acetaldehyde during oxidation of ethanol (H. L. Iversen and S. E. Damgaard, unpublished results).

Baker (1962) measured the effect of deuterium substitution into position 1 of ethanol and obtained an isotope effect upon ϕ_2 of about 2 at pH 8.6 and 27 °C. This value agrees fairly well with those obtained in this study (Figure 2).

Likewise, the $^{T}(V/K)$ value of 4.1 obtained by Palm et al. (1968) at pH 8.6 and 25 °C in the presence of 20 mM semicarbazide agrees well with the results of Figure 3. These authors also clearly demonstrated the importance of irreversibility by varying the concentration of semicarbazide in their assay. From the data of Plapp et al. (1973), a $^{D}(V/K)$ value of 2.3 may be calculated for the reaction with [U-^{2}H]ethanol at pH 8. This is slightly lower than expected from Figure 2.

At low concentrations of ethanol and at saturating concentrations of NAD$^+$, the kinetic state of the alcohol dehydrogenase mechanism named F-3 by Hanes et al. (1972) describes the kinetics of the reaction studied in this work. This kinetic state of their mechanism corresponds to the Theorell Chance mechanism supplemented with ternary complexes as reviewed by Cleland (1970). The mechanism is

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EO \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EOS \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ERP \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} ER \underset{k_{-5}}{\overset{k_5}{\rightleftharpoons}} E \quad (1)$$

The intrinsic primary isotope effect occurs at the interconversion of the central ternary complexes, k_3 and k_{-3}. E is apoenzyme, O is NAD$^+$ (or coenzyme analogue), S is ethanol (or isotopically substituted ethanol), R is NADH (or reduced coenzyme analogue), and P is acetaldehyde (or isotopically substituted acetaldehyde). When appropriate, the rate constants include the concentration terms to render them pseudo first order in the steady-state expressions. According to Northrop (1977), the $^{D}(V/K)$ isotope effect conforms to

$$^{D}(V/K) = \frac{^{D}k_3 + C_f + C_r\,^{D}K_{eq}}{1 + C_f + C_r}$$

where $^{D}k_3$ is the intrinsic isotope effect on the forward reaction under study and $^{D}K_{eq}$ is $^{D}k_3/^{D}k_{-3}$, the isotope effect upon the equilibrium of the reaction. The analogous expression for $^{T}(V/K)$ is obtained by exchanging the index D with T. When the dissociation of acetaldehyde as in this study is irreversible because the concentration of acetaldehyde is zero or when the analysis is based on measurement of the labeling pattern in acetaldehyde, as when $^{D}(V/K)$ is measured, then $C_f = k_3/k_{-2}$ and $C_r = k_{-3}/k_4$. When $^{D}K_{eq}$ is close to 1 or C_r is very small compared to $^{D}k_3$, this intrinsic isotope effect can be obtained according to Northrop (1977) from

$$\frac{^{D}(V/K) - 1}{^{T}(V/K) - 1} = \frac{^{D}k_3 - 1}{(^{D}k_3)^{1.44} - 1}$$

where $^{T}k_3$ in the denominator at the left side of the equation has been converted to $^{D}k_3$ according to Swain et al. (1958) [cf. also Stern & Vogel (1971)]. In the alcohol dehydrogenase reaction, $^{D}K_{eq}$ has been determined to be 1.07 (Cook et al., 1980), and hence, the error from this is quite small. Furthermore, it appears likely that C_r at most pH values is smaller than $^{D}k_3$ (Table IV). The data on $^{D}(V/K)$ and $^{T}(V/K)$ measured at various pHs and presented in Figures 2 and 3 have been used to calculate the intrinsic isotope effects $^{D}k_3$ and $^{T}k_3$, and these are shown in Table III. When the low values obtained at pH 6 are neglected, which are probably artifactual as discussed later, a constant intrinsic isotope effect for each coenzyme is obtained. When $^{D}k_3$ values of 9.93 with NAD$^+$, of 4.37 with thio-NAD$^+$, and of 5.5 with acetyl-NAD are used, the commitment function ($C_f + C_r\,^{D}K_{eq}$) may be calculated. These values are shown in Table IV.

Table IV: Calculated Values of $C_f + C_r\,^{D}K_{eq}$ from $^{D}(V/K)$ Values of Figure 2 and $^{D}k_3$ Values from Table III

	pH				
coenzyme	6	7	8	8.5	9
NAD$^+$	3.36	3.44	4.76	9.76	15.2
thio-NAD$^+$	0.58	0.67	0.75		3.16
acetyl-NAD$^+$	1.18	1.38	2.91		21.5

Both k_{-3} and k_4 are large (Shore & Gutfreund, 1970) and of about the same magnitude. C_r can therefore be expected to be quite small. A small value for C_r decreases the small error introduced by neglecting $^{D}K_{eq}$ when $^{D}k_3$ was calculated. Regarding the forward commitment, it is known that k_3 increases with pH, with an inflection point around pH 6.4 (Brooks et al., 1972). However, at values of pH above 7, where the net commitment shows the high sensitivity to pH, k_3 does only change very little. Its magnitude in this pH interval is about 140 s^{-1} (Shore & Gutfreund, 1970; Brooks & Shore, 1971; Shore et al., 1974). For k_{-2}, values have only been measured above pH 8.6, where Wong & Hanes (1973) obtained a value of 52.1 s^{-1}, which has been recalculated from their data by Cornish-Bowden & Wong (1978) to be 70.8 s^{-1}. At pH 9, a value of 20 s^{-1} has been found (Theorell & Tatemoto, 1972). With these values for the rate constants involved, C_f should be 2.1–2.3 at pH 8.6 and 7.5 at pH 9. A similar high sensitivity to pH around these values has been calculated in Table IV for the net commitment. The role of k_{-2} in the pH sensitivity of C_f is corroborated by the finding of a similar strong dependency of this rate constant with benzyl alcohol as ligand instead of ethanol (McFarland & Chu, 1975). In their study, the rate constant increased 6 times when pH was lowered from 8.75 to 8.2.

With the two coenzyme analogues used in this study, the intrinsic isotope effect is smaller than that with NAD$^+$. This implies that the commitment function with these analogues at pH 7–8 is also smaller that that with NAD$^+$ (Table IV). It is well-known that these exchanges of coenzyme causes the kinetic mechanism of undergo some qualitative changes, but apparently it still conforms to the reaction scheme discussed here (eq 1) (Shore & Theorell, 1967; Baici et al., 1975). Among these qualitative changes, the observations that the Dalziel parameters ϕ_2 and ϕ_2' become considerably increased are of relevance for the V/K isotope effect. With acetyl-NAD$^+$, both are increased by a factor of 10. According to Dalziel (1957) the expressions for ϕ_2 and ϕ_2' conform to

$$\phi_2 = \frac{k_{-2}k_4 + k_{-2}k_{-3} + k_4k_3}{k_2k_3k_4} = \frac{1/C_f + C_r/C_f + 1}{k_2}$$

$$\phi_2' = \frac{k_{-2}k_4 + k_{-2}k_{-3} + k_4k_3}{k_{-2}k_{-3}k_{-4}} = \frac{1/C_r + C_f/C_r + 1}{k_{-4}}$$

Simultaneous increases in both the numerators of the two expressions require decreases in both C_f and C_r, in agreement with the observed decrease in the net commitment, $C_f + C_r\,^{D}K_{eq}$ (Table IV). A concomitant decrease in k_2 or k_{-4} may contribute to this effect. In fact, Shore & Brooks (1971) observed that when NAD$^+$ was exchanged with acetyl-NAD$^+$, the concentration of ethanol needed to saturate the burst formation of acetyl-NADH in their stopped-flow analysis had to be increased considerably. That this effect may be related to a

decrease in k_2 is supported by binding studies with pyrazole (Shore & Gilleland, 1970). From the expressions for ϕ_2 and ϕ_2', it emerges that if k_{-4} remains unchanged then C_r is decreased considerably more than C_f by the exchange of coenzyme. On the other hand, lack of knowledge about the effect on k_{-4} prevents safe conclusions.

The decrease in $^Dk_3$, the intrinsic isotope effect, observed when NAD^+ is exchanged by one of the two coenzyme analogues may be related to the above-mentioned decrease in k_2 which is likely to reflect a change in the geometrical arrangement at the active site of the enzyme (Shore & Theorell, 1967), as this may result in a less linear transition state in the hydrogen transfer step (More O'Ferrall, 1970). Besides this possible explanation, the heat of reaction of the hydrogen or hydride transfer step is likely to be altered by the coenzyme substitution, and thereby the symmetry of the transition state as well as the isotope effect may be changed, as suggested by Hammond (1955) and Westheimer (1961) and supported by experiments for instance of Pryor & Kneipp (1971). The transition state can be expected to be nearly symmetrical with NAD^+ due to the high intrinsic isotope effect. With the analogues, the transition state may be less symmetrical, resembling for instance the substrates to a high degree with a concomitant decrease in the intrinsic isotope effect.

For $^T(V/K)$, the commitment function is the same as for $^D(V/K)$, provided a complete absence of acetaldehyde can be obtained. If this cannot be obtained satisfactorily, however, k_{-4} being a function of the acetaldehyde concentration has a finite value, and C_r takes the form $(k_{-3}/k_4)(1 + k_{-4}/k_5)$. C_r does not depend upon NADH, which actually accumulates in the experiments, but it does depend upon acetaldehyde, which must be present in a small concentration during the assay. That this concentration does not change during the assay period is evident from the constancy of the $^T(V/K)$ measured as a reaction proceeds, but it does, however, violate the use of Northrops method since C_r would differ from that of the deuterium experiments. The similarity in $^T(V/K)$ with semicarbazide or aldehyde dehydrogenase and the failure to increase $^T(V/K)$ by increasing a semicarbazide from 33 to 100 mM at pH 7 indicate that this error is small. k_{-4}/k_5 is, however, not of negligible magnitude, especially not when k_{-4} increases with decreasing pH (Dalziel, 1963). These facts could explain the decrease in $^T(V/K)$ when pH decreases from 7 to 6. This view is corroborated by the fact that the pH sensitivity of $^T(V/K)$ in this pH interval is less pronounced with the coenzyme analogues for which C_r due to a smaller value of k_{-3}/k_4 and a higher value of k_5 as discussed above is expected to increase less when k_{-4} increases. The determination of $^T(V/K)$ in the pH interval between 6 and 7 is therefore likely to be invalidated by the presence of acetaldehyde even in low steady-state concentrations, and hence, the intrinsic isotope effects calculated in this pH interval are likely to be in error. However, the constancy of the intrinsic isotope effect between pH 7 and 9 suggests that this error is only significant at pH below 7. The magnitude of the intrinsic isotope effect with the natural coenzyme is in the upper part of the semiclassical range for deuterium and tritium effects (Westheimer, 1961; Richards, 1970). This, however, is not without precedent since several reactions are known to exhibit similarly high deuterium isotope effects (Lewis, 1978; Klinman, 1978; Strittmatter, 1966). Intrinsic deuterium isotope effects of this reaction have earlier been estimated at room temperature (23–25 °C) to be about 5.2–6. These values were, however, obtained with [U-^{2}H]ethanol (Shore & Gutfreund, 1970; Brooks et al., 1972; Brooks & Shore, 1971). The use of [U-^{2}H]ethanol in these studies may explain the lower isotope effects obtained. The properties of the fully deuterated ethanol compound differ significantly from (1*R*)-[1-^{2}H]ethanol and may affect the rate of hydrogen transfer in other ways than by the primary isotope effect.

Acknowledgments

I wish to thank Dr. F. Lundquist for valuable advice and Anita Sommerfeldt, Lillian Lund Hansen, and Inge Damgaard for excellent technical assistance.

References

Anderson, L. E., & McClure, W. O. (1973) *Anal. Biochem. 51*, 173–179.
Arslanian, M. J., Pascoe, E., & Reinhold, J. G. (1971) *Biochem. J. 125*, 1039–1047.
Baici, A. Luisi, P. L., & Attanasi, O. (1975) *J. Mol. Catal. 1*, 223–244.
Baker, R. H., Jr. (1962) *Biochemistry 1*, 41–47.
Bergmeyer, H.-U. (1962) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., Ed.) pp 290–292, Verlag Chemie, Weinheim/Bergstr., Germany.
Bergmeyer, H.-U., & Möllering, H. (1970) in *Methoden der Enzymatischen Analyse*, 2nd ed. (Bergmeyer, H.-U., Ed.) pp 1478–1486, Verlag Chemie, Weinheim/Bergstr., Germany.
Blair, A. H., & Vallee, B. L. (1966) *Biochemistry 5*, 2026–2034.
Bonnichsen, R. (1962) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., Ed.) pp 285–287, Verlag Chemie, Weinheim/Bergstr., Germany.
Brooks, R. L., & Shore, J. D. (1971) *Biochemistry 10*, 3855–3858.
Brooks, R. L., Shore, J. D., & Gutfreund, H. (1972) *J. Biol. Chem. 247*, 2382–2383.
Bush, K., Shiner, V. J., Jr., & Mahler, H. R. (1973) *Biochemistry 12*, 4802–4805.
Büttner, H. (1965) *Biochem. Z. 341*, 300–314.
Cleland, W. W. (1970) *Enzymes, 3rd Ed. 2*, 1–65.
Cleland, W. W. (1975) *Acc. Chem. Res. 8*, 145–151.
Cook, P. F., Blanchard, J. S., & Cleland, W. W. (1980) *Biochemistry 19*, 4853–4858.
Cornish-Bowden, A., & Wong, J.-F. (1978) *Biochem. J. 175*, 969–976.
Cronholm, T. (1979) *Biochem. Biophys. Acta 583*, 253–260.
Dalziel, K. (1957) *Acta Chem. Scand. 11*, 1706–1723.
Dalziel, K. (1963) *J. Biol. Chem. 238*, 2850–2858.
Damgaard, S. E. (1977) *Biochem. J. 167*, 77–86.
Damgaard, S. E. (1980a) *Biochem. J. 191*, 613–618.
Damgaard, S. E. (1980b) XIth Annual Nordic Meeting on Biological Alcohol Research, Helsinki, Finland, Abstr. No. 2 (ISBN 951-9479-14-7).
Dworschack, R. T., & Plapp, B. V. (1977) *Biochemistry 16*, 2716–2725.
Gershman, H., & Abeles, R. H. (1973) *Arch. Biochem. Biophys. 154*, 659–674.
Gornall, A. G., Bardawill, C. J., & David, M. M. (1949) *J. Biol. Chem. 177*, 751–766.
Groves, W. E., Davis, F. C., Jr., & Sells, B. H. (1968) *Anal. Biochem. 22*, 195–210.
Grunnet, N. (1970) *Biochem. Biophys. Res. Commun. 41*, 909–917.
Grunnet, N. (1973) *Eur. J. Biochem. 35*, 236–243.
Hammond, G. S. (1955) *J. Am. Chem. Soc. 77*, 334–338.
Hanes, Ch. S., Bronskill, P. M., Gurr, P. A., & Wong, J. T.-F. (1972) *Can. J. Biochem. 50*, 1385–1413.

Klinman, J. P. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol. 46*, 415–494.
Lewis, E. S. (1978) in *Proton-Transfer Reactions* (Caldin, E. F., & Gold, V., Eds.) pp 317–338, Chapman and Hall, London.
Lundquist, F. (1962) in *Methoden der Enzymatischen Analyse* (Bergmeyer, H.-U., Ed.) pp 303–307, Verlag Chemie, Weinheim/Bergstr., Germany.
Markovič, O., & Theorell, H. (1972) in *Structure and Function of Oxidation Reduction Enzymes* (Åkeson, Å., & Ehrenberg, A., Eds.) pp 635–637, Pergamon Press, Oxford.
Markovič, O., Theorell, H., & Rao, S. (1971) *Acta Chem. Scand. 25*, 195–205.
McFarland, J. T., & Chu, Y.-H. (1975) *Biochemistry 14*, 1140–1146.
More O'Ferrall, R. A. (1970) *J. Chem. Soc. B*, 785–790.
Northrop, D. B. (1975) *Biochemistry 14*, 2644–2651.
Northrop, D. B. (1977) in *Isotope Effects on Enzyme-Catalyzed Reactions* (Cleland, W. W., O'Leary, M. H., & Northrop, D. B., Eds.) pp 122–152, University Park Press, Baltimore.
Palm, D., Fiedler, T., & Ruhrseitz, D. (1968) *Z. Naturforsh., B: Anorg. Chem., Org. Chem., Biochem., Biophys., Biol. 23B*, 623–628.
Plapp, B. V., Brooks, R. L., & Shore, J. D. (1973) *J. Biol. Chem. 248*, 3470–3475.
Pryor, W. A., & Kneipp, K. G. (1971) *J. Am. Chem. Soc. 93*, 5584–5586.
Rafter, G. W., & Colowick, S. P. (1957) *Methods Enzymol. 3*, 887–890.
Reynier, M. (1969) *Acta Chem. Scand. 23*, 1119–1129.
Richards, J. H. (1970) *Enzymes, 3rd Ed. 2*, 321–333.
Shore, J. D., & Theorell, H. (1967) *Eur. J. Biochem. 2*, 32–36.
Shore, J. D., & Gilleland, M. J. (1970) *J. Biol. Chem. 245*, 3422–3425.
Shore, J. D., & Gutfreund, H. (1970) *Biochemistry 9*, 4655–4659.
Shore, J. D., & Brooks, R. L. (1971) *Arch. Biochem. Biophys. 147*, 825–827.
Shore, J. D., Gutfreund, H., Brooks, R. L., Santiago, D., & Santiago, P. (1974) *Biochemistry 13*, 4185–4190.
Siegel, J. M., Montgomery, G. A., & Bock, R. M. (1959) *Arch. Biochem. Biophys. 82*, 288–299.
Stein, A. M., Lee, J. K., Anderson, C. D., & Anderson, B. M. (1963) *Biochemistry 2*, 1015–1017.
Stern, M. J., & Vogel, P. C. (1971) *J. Am. Chem. Soc. 93*, 4664–4675.
Strittmatter, P. (1966) in *Flavins and Flavoproteins* (Slater, E., Ed.) Vol. 8, pp 325–340, Elsevier, New York.
Swain, C. G., Stivers, E. C., Reuwer, J. F., Jr., & Schaad, L. J. (1958) *J. Am. Chem. Soc. 80*, 5885–5893.
Theorell, H., & Tatemoto, K. (1972) in *Structure and Function of Oxidation-Reduction Enzymes* (Åkeson, Å., & Ehrenberg, A., Eds.) pp 763–765, Pergamon Press, Oxford.
Ugarte, G., & Iturriaga, H. (1976) *Front. Gastrointest. Res. 2*, 150–193.
Westheimer, F. H. (1961) *Chem. Rev. 61*, 265–273.
Wong, J. T.-F., & Hanes, Ch. S. (1973) *Acta Biol. Med. Ger. 31*, 507–514.
Wratten, C. C., & Cleland, W. W. (1965) *Biochemistry 4*, 2442–2451.

Purification and Properties of Glutamine Phosphoribosylpyrophosphate Amidotransferase from *Bacillus subtilis*†

Joseph Y. Wong, David A. Bernlohr, Charles L. Turnbough,‡ and Robert L. Switzer*

ABSTRACT: A procedure for the rapid and efficient purification of glutamine phosphoribosylpyrophosphate amidotransferase to better than 98% homogeneity from derepressed *Bacillus subtilis* cells is described. The molecular weight of the subunit was estimated to be about 50 000. The purified enzyme exhibits microheterogeneity on electrophoresis on highly resolving polyacrylamide gels; it is suggested that this heterogeneity results from limited proteolytic modification of the native subunit. The native enzyme exists in equilibrium among tetrameric, dimeric, and monomeric forms. The influence of enzyme concentration and the presence of substrates and allosteric inhibitors on this equilibrium are described. There is no simple correlation between allosteric inhibition and stabilization of dimeric or tetrameric states. The amino acid composition of the amidotransferase is reported; presence of a 4Fe–4S center in the enzyme was described previously. Preparation of inactive apoprotein by treatment with 1,10-phenanthroline and general characteristics of the apoprotein are presented.

Glutamine phosphoribosylpyrophosphate amidotransferase (EC 2.4.2.14), hereafter called "amidotransferase", catalyzes the first reaction of purine nucleotide biosynthesis de novo and has received considerable attention as a major site of regulation of this pathway (Wyngaarden, 1972, 1973). Our attention to the amidotransferase of *Bacillus subtilis* grew out of the observation that the enzyme is inactivated in stationary cells in a reaction that requires O_2 (Turnbough & Switzer, 1975a). The enzyme is also oxygen labile in vitro (Turnbough & Switzer, 1975b). The site of reaction with oxygen in vitro was found to be a 4Fe–4S cluster covalently bound to the enzyme (Wong et al., 1977; Averill et al., 1980). The enzyme has thus become an object of considerable interest, from the perspective both of novel regulatory mechanisms and of novel functions

† From the Department of Biochemistry, University of Illinois, Urbana, Illinois 61801. *Received April 20, 1981.* This work was supported by U.S. Public Health Service Grant AI 11121 from the National Institute of Allergy and Infectious Diseases. Portions of this paper were taken from a thesis presented by J.Y.W. to the University of Illinois for the degree of Doctor of Philosophy in Biochemistry.
‡ Present address: Department of Microbiology, University of Alabama in Birmingham, Birmingham, AL 35294.

0006-2960/81/0420-5669$01.25/0