In another experiment, when [α-^{2}H]alanine was oxidized to pyruvate or to acetate both in H_2O and in ^2H_2O, the signals of the methyl groups of these products were also singlet. This indicates that the intramolecular transfer of a hydrogen atom (from the α- to β-carbon in this case) is not involved in the enzymic oxidation of the amino acid, in contrast to the other reactions which are catalyzed by B_{12} enzymes[5,6].

In the case of L-amino acid oxidase, it has already been shown that a hydrogen–deuterium exchange does not occur at the β-carbon of the substrate[7]. Since it has recently been shown that L-amino acid oxidase has a similar reaction mechanism to D-amino acid oxidase[8], it may be expected analogically that the intramolecular transfer of a hydrogen atom does not occur in the L-amino acid oxidase reaction either.

From these results, it is concluded that the participation of the group at the β-position of the substrate is not involved in the D-amino acid oxidase reaction and that the occurrence of an α,β-unsaturated intermediate is ruled out.

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The ternary complexes of fatty acid amides with horse liver alcohol dehydrogenase and NAD$^+$

Stable ternary complexes of fatty acid amides, NADH and horse liver alcohol dehydrogenase (alcohol:NAD$^+$ oxidoreductase, EC 1.1.1.1) were first reported by WINER AND THEORELL[1]. At that time, no evidence was obtained in their equilibrium fluorescence titrations for the existence of the corresponding ternary complexes composed of alcohol dehydrogenase, NAD$^+$ and amides. The failure to demonstrate alcohol dehydrogenase–NAD$^+$–amide complexes suggested an absolute specificity

Abbreviations: PHMB, *p*-hydroxymercuribenzoate; $K_{EO,I}$, the dissociation constant of ternary complex into alcohol dehydrogenase–NAD$^+$ and free amide; $K_{ER,I}$, the dissociation constant of ternary complex into alcohol dehydrogenase–NADH and free amide.

of alcohol dehydrogenase–NADH for amide binding and indicated the substrate binding site for alcohol dehydrogenase–NADH is markedly different from that for alcohol dehydrogenase–NAD^+. This latter conclusion is difficult to rationalize with considerations of microscopic reversibility for the alcohol dehydrogenase reaction[2] and is in conflict with other data on ternary complexes composed of enzyme, coenzyme, and ethanol and imidazole[3,4].

The present communication describes direct evidence for the formation of alcohol dehydrogenase–NAD^+-amide ternary complexes under equilibrium conditions and indicates that there is no absolute specificity with respect to amide binding for either type of enzyme–coenzyme binary complex but only a relative difference in affinity. Some kinetic evidence based on the mixed inhibition observed with amides in Lineweaver–Burk plots has suggested the existence of alcohol dehydrogenase–NAD^+–amide ternary complexes[6,3].

Horse liver alcohol dehydrogenase (Worthington or Boehringer) was dialyzed against 4×1000 ml of 0.05 M phosphate buffer (pH 7.0) for 3 days at 4°. Enzyme concentrations were determined fluorimetrically by titration with NADH in the presence of 0.1 M isobutyramide[1]. β-NAD^+ (Sigma Chemical Co.) was purified by DEAE-cellulose chromatography[7]. Since alcohol dehydrogenase crystallized from 6–10% ethanol is difficult to free from trace amounts of ethanol even after exhaustive dialysis, 1 mM pyruvate and catalytic amounts of lactate dehydrogenase were added to solutions of NAD^+ and alcohol dehydrogenase to reoxidize any NADH that might have been formed by oxidation of the residual ethanol.

A number of lines of evidence establish that the alcohol dehydrogenase–NAD^+–amide complexes exist and are distinct from the alcohol dehydrogenase–NADH–amide complexes. For example, the fluorimetric and spectrophotometric spectra of the alcohol dehydrogenase–NAD^+–*n*-butyramide complexes can be readily distinguished from those of the alcohol dehydrogenase–NADH–*n*-butyramide complexes. The NAD^+ complex possesses a fluorescence emission maximum at 385 mμ while the NADH complex has an emission maximum at 420 mμ when excited at 320 mμ (Fig. 1). The absorption spectrum of the NAD^+ complex possesses a long wavelength absorption maximum at 315 mμ while the NADH complex has its maximum at 325 mμ. The extinction coefficients of the alcohol dehydrogenase–NAD^+–amide complexes at 315 mμ, for all three amides tested, are approximately $6 \cdot 10^3$ $cm^{-1} \cdot M^{-1}$.

The two types of complexes also differ with respect to their behavior with pyruvate and lactate dehydrogenase. The emission spectrum of alcohol dehydrogenase–NAD^+–*n*-butyramide is stable in the presence of pyruvate and lactate dehydrogenase while that of the alcohol dehydrogenase–NADH–*n*-butyramide ternary complex is not. When pyruvate and lactate dehydrogenase is added to the latter complex at pH 7, the fluorescence emission at 420 mμ is slowly decreased and after 20 min, a fluorescent peak at 385 mμ, characteristic of the alcohol dehydrogenase–NAD^+–amide complex, appears. Similar results are obtained when acetaldehyde in high concentrations (10 mM) is substituted for pyruvate and lactate dehydrogenase.

Differences in the reactivity of the two complexes with respect to *p*-hydroxymercuribenzoate (PHMB) can also be demonstrated. The addition of PHMB (0.1 mM) to a fully saturated alcohol dehydrogenase–NADH–isobutyramide complex quenches the fluorescence emission at 420 mμ with the immediate appearance of a new fluorescence maximum at 465 mμ, characteristic of free NADH. When the same concen-

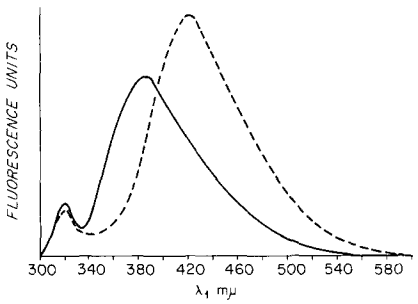

Fig. 1. Fluorescence emission spectra of alcohol dehydrogenase–NAD$^+$–*n*-butyramide (———) and alcohol dehydrogenase–NADH–*n*-butyramide (— — —). ———, [alcohol dehydrogenase] = 3.70 μN, [NAD$^+$] = 0.223 mM, [*n*-butyramide] = 8.93 mM, [lactate dehydrogenase] = 10 mμM, [pyruvate] = 0.892 mM; — — —, alcohol dehydrogenase] = 3.69 μN, [NADH] = 4.36 μM, [*n*-butyramide] = 8.92 mM. $\lambda_{excitation}$ = 320 mμ; 0.05 M phosphate buffer (pH 7.0).

tration of PHMB is added to a fully saturated alcohol dehydrogenase–NAD$^+$–isobutyramide complex, the quenching of fluorescence at 385 mμ is considerably slower and is not fully quenched even after 10 min.

A further characteristic of the alcohol dehydrogenase–NAD$^+$–amide complexes is their relatively slow rate of formation. The half life for formation of the *n*-butyramide complex is about 1 min in a solution of 0.102 mN alcohol dehydrogenase, 0.32 mM NAD$^+$, and 1.07 mM *n*-butyramide. The corresponding complex containing NADH is totally formed in less than 30 sec under comparable conditions.

Dissociation constants of the alcohol dehydrogenase–NAD$^+$–amide ternary complexes were determined graphically by the method of WORONICK[8]. Table I lists the dissociation constants determined for the alcohol dehydrogenase–NAD$^+$–amide complexes for several amides and compares them with those previously found for the corresponding ternary complexes with NADH. Notable is the similar dependence of the dissociation constants on chain length in both types of complexes.

The slow rate of formation of the alcohol dehydrogenase–NAD$^+$–amide complexes and the magnitude of the dissociation constant make the kinetic importance of these complexes in initial velocity studies depend strongly on the amide concentration. As noted above, WORONICK[6] and THEORELL AND MCKINLEY-MCKEE[3] have postulated the existence of alcohol dehydrogenase–NAD$^+$–amide complexes on the

TABLE I

Amide	$K_{EO,I}$ *(mM)* (fluorimetrically)*	$K_{EO,I}$ *(mM)* (spectrophotometrically)*	$K_{ER,I}$ *(mM)** (fluorimetrically)*
n-Butyramide	0.81	1.16	0.064 (ref. 1)
Isobutyramide	7.9	8.2	0.140 (ref. 4)
Hexamide	0.75	0.85	0.011 (ref. 1)

* Temp. 26°, 0.05 M phosphate (pH 7.0).

** $K_{ER,I}$ = dissociation constant of ternary complex into alcohol dehydrogenase–NADH and amide.

basis of initial rate measurements carried out in the presence of high concentration of amides. From their kinetic data, the latter authors suggested the alcohol dehydrogenase–NAD^+–isobutyramide complex had a $K_{EO,I}$ (dissociation constant of ternary complex into alcohol dehydrogenase–NAD^+ and free amide) of about 5 mM. This estimated value of $K_{EO,I}$ compares favorably with the constants obtained in the present experiments as determined spectrophotometrically as well as fluorimetrically.

The demonstration that amides can form ternary complexes with liver alcohol dehydrogenase and either NAD^+ or NADH indicates that there is no absolute specificity with respect to amide binding for either type of enzyme–coenzyme binary complex but only a relative difference in affinity as reflected in the dissociation constants reported in Table I. The existence of two types of amide complexes further suggests that there is only a single substrate binding site in both the alcohol dehydrogenase–NAD^+ and alcohol dehydrogenase–NADH complexes and not two distinct sites for alcohol and aldehydes substrates as has been previously postulated[1,9].

The chemical structure of the alcohol dehydrogenase–NAD^+–amide complex is as yet unknown. Trifluoroacetamide, trimethylacetamide, benzamide, dimethylacetamide and dimethylbutyramide do not form ternary complexes with the characteristic fluorimetric and spectrophotometric properties observed with *n*-butyramide, *n*-hexamide, and isobutyramide under comparable conditions.

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