Structural Changes in the Phospholipid Regions of the Axonal Membrane Produced by Phospholipase C Action*

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ABSTRACT: Phospholipase C cleaves most of the phospholipids in plasma membranes, rendering the fatty acid chains more mobile. We used spin labels to study the effect of phospholipase C action on the molecular motion of the phospholipids in nerve membranes. A substantial amount of bilayer character survives phospholipase C treatment, suggesting that membrane lipids can maintain their ordered structure in the absence of ionic interactions involving the phospholipid head group.

This paper describes the use of spin labels to assess the importance of ionic interactions involving phospholipid head groups in maintaining membrane structure. Nuclear magnetic resonance (nmr) studies showed that the lipid regions of erythrocyte membranes become more mobile after treatment with phospholipase C, with no detectable change in the helical content of membrane proteins (Glaser *et al.*, 1970). Hubbell and McConnell (1969) obtained electron spin resonance (esr) spectra from fatty acid spin labels imbedded in the membranes of the walking leg nerve axon of the Maine lobster, *Homarus americanus*. From a detailed analysis of these spectra they concluded that the axolemma contains phospholipid bilayer regions perpendicular to the axonal axis. We have used their technique to investigate the fate of this bilayer structure in the axonal membranes of *H. americanus* after removing the polar head groups of the phospholipids with phospholipase C.

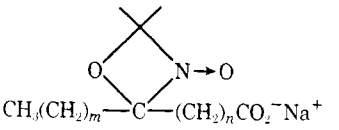
Materials and Methods

Walking leg nerve bundles (3–4 cm long; 1–3 mm in diameter) of the lobster *H. americanus* were exised and separated from the accompanying connective tissue. All further operations were then performed in lobster–Ringers solution (Mendelson, 1969) at 4°.

A phospholipase C solution was made up by homogenizing phospholipase C (Sigma Chemical Co., St. Louis, Mo.) from *Clostridium welchii* at a concentration of 0.5 mg/ml (5 units/ml) in lobster–Ringers solution, followed by heating at 60° for 5 min and finally centrifugation at 100,000*g* for 1 hr. Ten to twenty enzyme units were then agitated over a nerve bundle (containing about 2 mg of total protein) for 1 hr at 37°. The enzyme preparation was found to possess no proteolytic activity, as determined by negligible release of ninhydrin-positive material after treatment of red blood cell membranes. It was also found that the membrane protein polyacrylamide gel electrophoresis pattern (performed as described by Lenard, 1970) remained unaffected by enzyme treatment.

Total protein and total phosphorus were determined by the methods of Lowry *et al.* (1951) and Bartlett (1959), respectively. Thin-layer chromatography on silica gel H and the quantitation of the separated phospholipids was determined as described by Simpkins *et al.* (1971). Cholesterol determinations were performed according to Abel *et al.* (1952).

The fatty acid spin labels



employed were the 12-NS[1] (12-NS; $m = 5$, $n = 10$), 7-NS (7-NS; $m = 10$, $n = 5$), and the 5-NS (5-NS; $m = 12$, $n = 3$). The 12-NS was synthesized according to Waggoner *et al.* (1969) and the 5-NS from the 5-keto acid prepared according to Jones (1947). The 7-NS was prepared from the 7-ketostearic acid prepared according to Hunig and Eckardt (1962), as modified by O. H. Griffith (personal communication, 1970).

A solution of the fatty acid spin labels in ether was evaporated in a beaker, absorbed into 5% BSA (fatty acid poor, Calbiochem) in lobster–Ringers solution and then exchanged into the treated and control nerve bundles for 1 hr at 4°. The nerves were then washed exhaustively with Ringers solution. The esr spectra were obtained with a Joelco MES-ME-1X spectrometer with a tissue cell at 20–25°.

Electron microscopy was performed with an Hitachi HU 12. Specimens were fixed in 1% glutaraldehyde for 2 hr, followed by 1% osmium tetroxide, dehydrated by an alcohol series, and sections were poststained with saturated uranyl acetate–Reynolds solution.

Results

In order to determine the extent of the action of phospholipase C, the nerves were homogenized in lobster–Ringers and the total protein and total phosphorus determined according to Lowry *et al.* (1951) and Bartlett (1959), respectively. The homogenized nerve bundles were then extracted three times with chloroform methanol (2:1, v/v) in order to determine what percentage of the total phosphorus was lipoid in origin. The results in Table I show that both the total phosphorus and the lipid phosphorus (*i.e.*, phosphorus extractable with chloroform methanol) were decreased on enzyme treatment, being reduced to about 20–30% of the untreated control value. This

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[1] Abbreviations used are: NS, nitroxide stearate; BSA, bovine serum albumin.

TABLE I: Phosphorus Determinations of Homogenized Nerve.[a]

Units of Phospholipase C Employed/ Nerve Bundle	Total Phosphorus/ mg of Total Protein	P Present in Chloroform–Methanol Extract/mg of Initial Total Protein	% of Total Phospholipid Cleaved by Enzyme (%)
0	32 ± 1	19 ± 1	0
9	18	6	68 ± 5
18	15	6	68 ± 5

[a] The values are expressed as micrograms of phosphorus per milligram of proteins (two determinations). The total phosphorus values change for each nerve preparation depending on the amount of endogenous protein and phosphorous. The percentage decrease of phosphorous present in the lipid extract after enzyme treatment however is always 70–80%.

means that if all the membrane phospholipid can be extracted, then about 70–80% of the lipid phosphorus is released by the phospholipase C treatment. These nerve bundles are not solely made up of axons; they also contain some Schwann cell, connective tissue, and axoplasm. However, as can be seen from the morphological investigation by De Lorenzo *et al.* (1968), the amount of axonal membrane present is far greater than membrane material from these other organelles, and so it was assumed that total phospholipid determinations presented a realistic idea as to the extent of enzyme digestion of the axolemma. The chloroform–methanol (2:1, v/v) lipid extracts were also used to determine whether loss of cholesterol occurred and which phospholipids had been cleaved after enzyme treatment. It should be emphasized that the nerve preparation contained axoplasm and variable but small amounts of cells other than those of the axolemma. Thus as can be seen from Figure 1, all of the phospholipids were affected by enzyme treatment although a small amount of phosphatidylserine and -ethanolamine was found to remain with the treated nerve bundles. However, the lysophosphatidyl compounds appeared unaffected. The main product of the enzymatic cleavage was diglyceride which migrated at the solvent front. Also the concentration of cholesterol did not appear to decrease, in fact, in some experiments an increase was found, presumably due to loss of protein from the axoplasm.

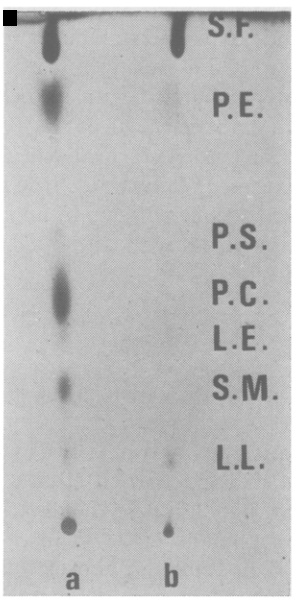

FIGURE 1: Photograph of a silica gel H thin-layer chromatograph plate sprayed with sulfuric acid (50% w/w) for visualization of the phospholipids. Control phospholipids are presented in column a and phospholipase C treated membrane phospholipids in column b. The following phospholipids were separated: LL, lysolecithin; SM, sphingomyelin; LE, lysoethanolamine; PC, phosphatidylcholine; PS, phosphatidylserine; PE, phosphatidylethanolamine. Diglyceride and cholesterol migrated with the solvent front (SF).

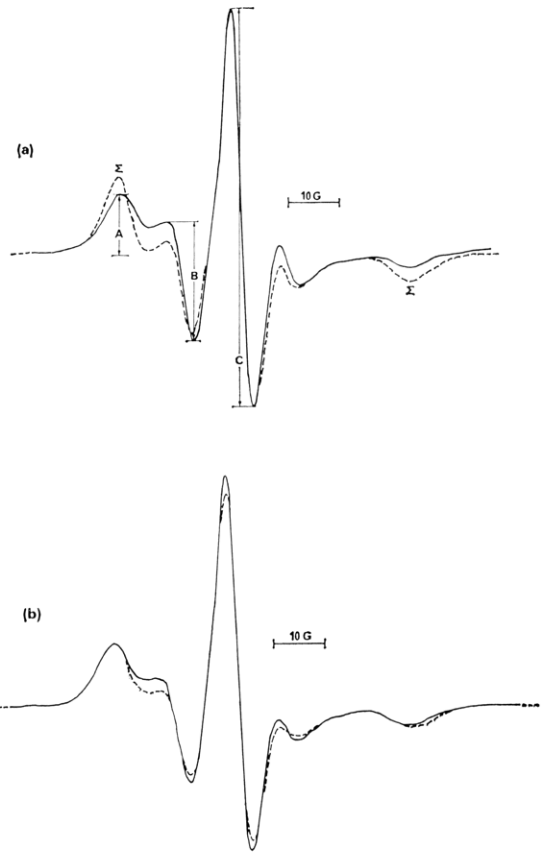

FIGURE 2: (a) The five nitroxide stearate spin label in the axonal membrane with the axonal axis parallel (—) and perpendicular (----) to the applied magnetic field; (b) the phospholipase C treated nerve parallel (—) and perpendicular (---) to the applied field.

Electron spin resonance spectra of the spin-labeled nerves were obtained with the axonal axis parallel and perpendicular to the applied magnetic field H. A spectrum of the nerve (Figure 2a) can be divided into three amplitudes A, B, and C—these were measured for each spectrum. The spectra for the 5-NS-labeled nerve are presented in Figure 2a and for the enzyme-treated nerve in Figure 2b. The motion of the 5-NS spin label is about the long axis of the fatty acid chain and results in an outer hyperfine doublet (labeled Σ in Figure 2a). This doublet can also be resolved in the case of the 7-NS spin-labeled nerve (Figure 3a), but not with the 12-NS nerve (Figure 4a) where the nitroxide group is located too far from its charged head group for its motion to become anisotropic. By observing the resonance with the axonal axis parallel and perpendicular to the applied magnetic field, it can be seen that the amplitude of the outer doublet decreases when the field is

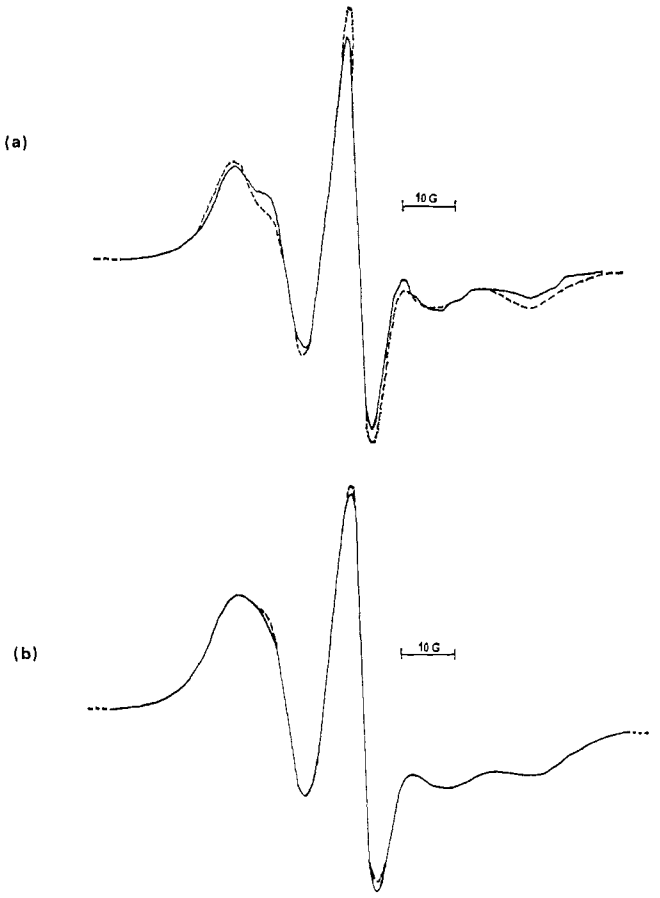

FIGURE 3: (a) The seven nitroxide spin label in the axonal membrane parallel (—) and perpendicular (---) to the applied field; (b) the seven nitroxide stearate in enzyme-treated nerve, parallel (—) and perpendicular (----) to the applied field.

parallel to the axonal axis. The preferred axial direc ion is therefore perpendicular to the applied field. The difference in the amplitude ratio of A to B between a nerve in the parallel and the perpendicular position gives a measure of the structural anisotropy of the nerve which has been suggested to be related to its bilayer character (Hubbell and McConnell, 1969). The 12-NS spectra presented in Figure 4a,b show no anisotropic effect but solely an increase in the mobility of the nitroxide group accompanying phospholipase C action. The 7-NS which possesses a nitroxide at 8 to 9 Å from its charged carbonyl group (measured with a Corey–Pauling–Koltun molecular model kit) is fixed firmly enough by this charged head group to give rise to the outer hyperfine doublet, and it can be seen from Figure 3a that the untreated nerve exhibits anisotropy, whereas the treated nerve (Figure 3b) does not. In the case of the 5-NS label with the nitroxide group situated at about 6 Å from the charged carbonyl group, greater differences are apparent in the parallel and perpendicular spectra. These spectral differences (anisotropy) are decreased in the enzyme treated nerve but are still present. In Table II is presented the A:B ratio for each spectrum and the ratio of this amplitude ratio between the parallel and perpendicular spectra (θ). This parameter (θ) gives a measure of the anisotropy present in each nerve and is unity for an isotropic situation. It can be seen that as expected the 5-NS spin-labeled nerve (θ = 1.6) exhibits more anistropy than the one labeled with the 7-NS (θ = 1.2). Enzyme treatment of the nerve results in the disappearance of anisotropy in the case of the 7-NS (θ = 1.0),

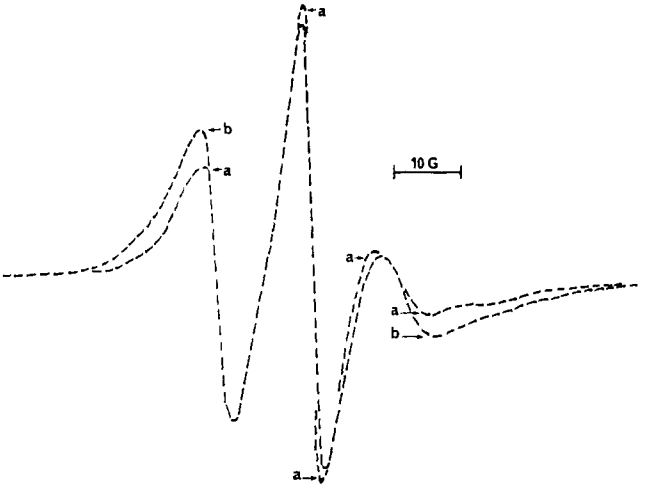

FIGURE 4: (a) The twelve nitroxide stearate in the axonal membrane perpendicular to the magnetic field; (b) the same label in phospholipase C treated nerve perpendicular to the magnetic field.

but not in the case of the 5-NS-labeled nerve where the anisotropy remaining *after treatment* (θ = 1.2) is the same as that shown in the *untreated* 7-NS-labeled nerve (θ = 1.2). It should be stressed that phospholipase C treatment resulted in 70–80% cleavage of the lipid head groups. Electron micrographs presented in Figure 5 show that the number of axons per unit field decrease as expected from the swelling observed by the naked eye. The vesicular structure is also present to a greater degree in the treated nerve preparation; however, the axons are still found to exist as structural entities.

Discussion

From the data presented in Table I, it can be assumed that a significant proportion (approximately 70%) of the phospholipid head groups were cleaved by the phospholipase C treatment. From Table II, it is obvious that the anisotropy present with a 5-NS-labeled nerve is decreased by enzyme treatment

TABLE II: Anisotropy Exhibited by Spin-Labeled Lobster Nerves.[a]

Nerve and Spin Label Employed	A/B	θ
Control 5-NS		
Perpendicular	0.85	1.6 ± 0.1
Parallel	0.52	
Treated 5-NS		
Perpendicular	0.78	1.2 ± 0.1
Parallel	0.63	
Control 7-NS		
Perpendicular	0.78	1.2 ± 0.1
Parallel	0.61	
Treated 7-NS		
Perpendicular	0.70	1.0 ± 0.1
Parallel	0.67	

[a] The ratio A/B was calculated from each individual spectrum. The anisotropy factor (θ) is the ratio of (A/B) perpendicular: (A/B) parallel.

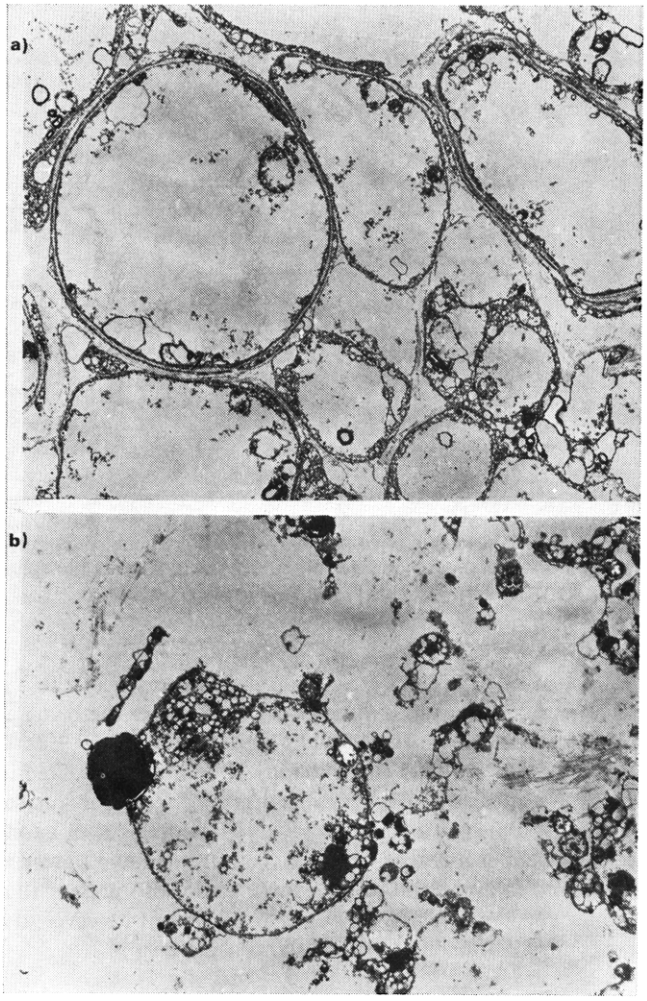

FIGURE 5: Electron micrographs of a transverse section of the (a) control nerve bundle and (b) the treated nerve bundle (overall magnification 7200×). The lower micrograph which is a typical field of the treated nerve preparation shows only one to two axons per field in contrast to the control (see upper photograph) and is a result of the swelling produced by the enzyme treatment. The treated axons have more vacuolated structures and more electron dense material associated with them than the untreated controls, but they still exist as structural entities.

and is, in fact, abolished in the case of the 7-NS. It is reasonable to assume for the reasons outlined by Hubbell and McConnell (1969) that the anisotropy seen here is very similar to that which should be observed with a lipid bilayer. It therefore appears that, even after removal of 70% of the lipid phosphorus and cleavage of the ionic head groups from all the phospholipids present in the nerve bundle preparations, a significant amount of bilayer character remains in the lipid regions of the membrane. How the lipid remains in this high state of structural organization is open to question. Protein–lipid interactions may be the dominant factor, or perhaps the lipid regions are stable enough by themselves *via* hydrophobic interactions of the fatty acid chains. Both possibilities suggest that the phospholipids rely very little on ionic interaction of their charged head groups for the stability of an ordered membrane structure.

The nerve bundle after enzyme treatment swells, but the axis of the nerve bundle still remains the same as observed by the naked eye. The gross morphology as determined by electron microscopy did not appear greatly altered. The number of electron dense bodies separate from the membranes which are suggested by Coleman *et al.* (1970) to be neutral lipid produced by the enzyme treatment did not significantly increase. Also lobster nerve preparations treated with phospholipase C still conduct action potentials (Rosenberg, 1970). The assumption that enzyme treatment does not destroy the axonal membrane in terms of its overall structure appears therefore justifiable.

If a comparison is made between the θ value of the 5-NS spin-labeled nerve before and after enzyme treatment, it appears that a substantial amount of the bilayer character remains even with 70% of the lipid phosphorus cleaved. From the data obtained with the 7- and 12-NS spin-labeled nerves, it can be seen that the membrane core becomes far more fluid after removal of the lipid head groups, and so the effect of enzyme treatment is transmitted transversely through the whole of the axonal membrane.

A tacit assumption of the spin-label study has been that the fatty acid spin labels exchange randomly with the lipid regions in the axonal membrane. If, for some reason, the spin labels have diffused predominantly into phospholipid regions where the phospholipid head groups have not been cleaved (N.B. 20–30% of the membrane phosphorus remains uncleaved), then the conclusions made here are debatable. In order to check this criticism, we have labeled red blood cell membranes and these nerve bundle preparations, both before and after the phospholipase C treatment and compared the esr spectra, which were identical. Unless rearrangement of the spin label in the membrane labeled *before* the reaction with phospholipase C is possible *after* enzyme treatment, which is doubtful, it appears justifiable to assume that the spin label diffuses randomly into the lipid regions.

We therefore conclude that phospholipase C treatment produces an increase in fluidity throughout the whole of the lipid regions of the axonal membrane accompanied with loss of a major proportion of the membrane bilayer character. However, a minor but significant amount of bilayer character still remains in the treated lipid regions suggesting that the diglyceride–phospholipid lattice can still exist as an ordered array without ionic interaction between the phospholipid head groups.

Acknowledgment

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Effect of Substrate Structure on the Rate of the Catalytic Step in the Liver Alcohol Dehydrogenase Mechanism*

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ABSTRACT: The catalytic step of the oxidation of alcohols by liver alcohol dehydrogenase has been resolved for several substrates by employing deuterated analogs using transient and steady-state kinetic techniques. With the two primary alcohols, ethanol and propanol, a presteady-state burst of liver alcohol dehydrogenase-bound NADH was observed. Substantial kinetic isotope effects on the rate of this burst were taken as evidence that this burst was the catalytic step. Methanol and 2-propanol did not produce a burst but only steady-state turnover which was, as had been previously reported, slower than the rate constant for dissociation of liver alcohol dehydrogenase bound NADH. Isotope effects on the turnover numbers at maximum velocity allowed the assignment of the catalytic step as rate limiting for these substrates. Large differences in catalytic rates were observed between the four substrates investigated. These differences are discussed with regard to the relative importance of steric effects, hydrophobic binding, and molecular orientation.

Several attempts have been made, using steady-state kinetics, to investigate the relationship between substrate structure and the rate of catalysis by liver alcohol dehydrogenase. These studies have been complicated due to the ordered reaction mechanism of the enzyme (Theorell and Chance, 1951) with the dissociation rate of binary enzyme–product coenzyme complex being rate limiting at maximum velocity (Dalziel, 1963). In an attempt to study the catalytic mechanism, Blomquist (1966) related the structure of substituted benzaldehydes to ϕ_2 values (Dalziel, 1957b). These values, however, are complex functions of the binding of substrate and release of product as well as the catalytic interconversion of ternary complexes. Dalziel and Dickinson (1966a, 1967) reported that several secondary alcohols exhibited maximum velocities considerably slower than primary alcohols, in which the dissociation rate of NADH[1] from binary enzyme–coenzyme complex is rate limiting. Despite the complex scheme required to explain results with secondary alcohols, they surmized that the maximum velocity for these substrates was limited by the rate of interconversion of ternary complexes.

Shore and Gutfreund (1970) recently reported the isolation of the hydride-transfer step in the LADH mechanism employing the use of deuterated ethanol and transient kinetic techniques. They reported the existence of a kinetic isotope effect of 6 on the rate constant for the initial formation of dehydrogenase-bound NADH. This substantial isotope effect was taken as evidence that the burst of bound NADH production was the hydride-transfer step or was controlled by the hydride-transfer rate. The primary objective of the present study is the determination of the effect of substrate structure on the rate of the catalytic step of the LADH mechanism. One secondary and three primary alcohols and their deuterated analogs were investigated, resulting in the discovery of large differences in rate constants for hydrogen transfer from these substrates to NAD^+.

Materials and Methods

Crystalline LADH was prepared from horse liver by the method of Theorell *et al.* (1966). The enzyme assay method of Dalziel (1957a) was used in conjunction with the NADH titration of enzyme in the presence of isobutyramide (Theorell and McKinley-McKee, 1961) to determine enzyme concentration. Coenzymes were purchased from either Boehringer Corp. or Sigma Chemical Corp., and NAD^+ was further purified by the procedure of Dalziel and Dickinson (1966b). Deuterioethanol and *d*-4-methanol were purchased from International Chemical and Nuclear Corp., Irvine, Calif.; deuterioisopropyl alcohol from Diaprep, Inc., Atlanta, Ga., and 1,1-dideuterio-*n*-propyl alcohol was synthesized using the method of Hill *et al.* (1952).

Initial rate studies for the determination of ϕ_0, the reciprocal of the turnover number, were made at saturating concentrations of NAD^+, greater than 1 mM, using an Eppendorf photometer adapted for fluorometry. Stopped-flow kinetic studies were performed on a Durrum-Gibson instrument which had a dead time of 2.5 msec. All kinetic studies were made at 25° in pH 7 phosphate buffer, ionic strength 0.1. First-order rate constants for the initial burst of bound NADH formation were calculated employing the Guggenheim method (Frost and Pearson, 1965) using only the initial exponential part of the rate curve or the method of Gutfreund and Sturtevant (1956) in which the steady-state portion was algebraically subtracted. Both methods of calculation resulted in the same numerical values for the rate constants.

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[1] Abbreviations used are: NAD^+ and NADH, oxidized and reduced nicotinamide-adenine dinucleotide; LADH, liver alcohol dehydrogenase.