Transients in the Reactions of Liver Alcohol Dehydrogenase*

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ABSTRACT: Transient intermediates in the liver alcohol dehydrogenase reaction mechanism were investigated using rapid kinetic techniques. In the presence of NaCl, the difference between V_{max} per equivalent of enzyme and the directly determined dissociation rate of NADH provided evidence for an additional rate-determining step. This step, with a rate constant of 11 sec^{-1}, represents an isomerization of enzyme–NADH complex. Studies of the rate of formation of enzyme-bound NADH demonstrated that at saturating concentrations of NAD^+ and ethanol this rate was 130 sec^{-1}. With saturating concentrations of deuterioethanol the rate was 25 sec^{-1} indicating that this step is either the hydride transfer or is controlled by the hydride-transfer rate. The results of this investigation have provided information on two previously unresolved steps in the liver alcohol dehydrogenase reaction mechanism. In addition, general guide lines for the study and interpretation of transients in dehydrogenases have been outlined.

The initial kinetic investigation of the liver alcohol dehydrogenase (LADH)[1] reaction (Theorell and Chance, 1951) utilized both steady-state and transient kinetic techniques. It resulted in the proposal of an ordered reaction mechanism with coenzyme combining first, no distinguishable ternary complexes, and the rate of coenzyme dissociation as the rate-limiting step at saturating concentrations. Subsequent steady-state kinetic studies (Theorell and McKinley-McKee, 1961a; Dalziel, 1963; Wratten and Cleland, 1963) firmly established the ordered reaction mechanism for LADH between pH 6.0 and 9.0. Although these investigations did not provide information on intermediate steps, product inhibition studies (Wratten and Cleland, 1963; Shore and Theorell, 1966) proved the existence of ternary complexes in the mechanism.

Thus far, very few studies on transient intermediates in NAD-linked dehydrogenases have been reported. Iwatsubo and Pantaloni (1967) showed that the production of NADPH from $NADP^+$ in the glutamate dehydrogenase reaction proceeded in two steps: a burst equal to the concentration of enzyme active centers followed by a slow reaction equal to the turnover number. Heck, McMurray, and Gutfreund (1968) used stopped-flow methods to demonstrate that the dissociation of NADH from lactic dehydrogenase was faster than the catalytic center activity at pH 6.0, indicating the existence of an isomeric enzyme–NADH complex. Criddle, McMurray, and Gutfreund (1968) used stopped-flow and temperature-jump techniques at alkaline pH to isolate a step in the lactic dehydrogenase reaction involving conversion of the isomeric ternary enzyme–NADH–pyruvate complex. Bernhard *et al.* (1970) have studied transients of LADH reactions with aromatic aldehydes by analyzing the burst of NADH oxidation.

The following observations of transients, relaxations, and partial reactions can be used to determine the rate constants of the individual steps of the reactions of dehydrogenases with their specific substrates. (1) At saturating concentrations of both substrates, the time course of the formation of NADH, enzyme bound and free, during the transient period before the steady state is reached, is a measure of the rate of interconversion of the ternary complexes. This process can include isomerization as well as hydride-transfer steps. Experiments with deuterium-labeled substrates may indicate whether hydride transfer is rate limiting for the ternary complex interconversion. The steady-state concentration of enzyme-bound NADH can also be evaluated from the study of initial transients. This in turn enables one to calculate the ratio of the rates of formation of enzyme-bound NADH to that of the dissociation of NADH from its enzyme complexes. In theory, it is possible to obtain additional information from transient experiments at low substrate concentrations but this approach has not yet been used in the study of enzyme reactions with two substrates. (2) Observation of the relaxation times of dehydrogenases with an equilibrium mixture of substrates can give information about the rates of interconversion of intermediates. The identification of specific relaxation times with particular elementary steps of the reaction depends on their concentration dependence and the spectral changes. Even before identification is achieved, the results of preliminary experiments permit statements about the minimum number of distinct steps. (3) Several partial reactions can be isolated and studied by flow and relaxation methods. If a spectral change occurs during nucleotide binding, this reaction can be investigated. The first-order reaction of stoichiometric E^{NADH} complex with excess substrate is another partial reaction which can be studied.

The results of the present study represent the application of these techniques to the LADH reaction mechanism. Although only preliminary investigations are reported, it has been possible to discern several new intermediate steps in the mechanism.

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[1] The following abbreviations have been used: LADH or E, liver alcohol dehydrogenase; NAD^+ or O, oxidized nicotinamide–adenine dinucleotide; NADH or R, reduced nicotinamide–adenine dinucleotide; $K_{E,R}$, the dissociation constant of binary LADH–NADH complex.

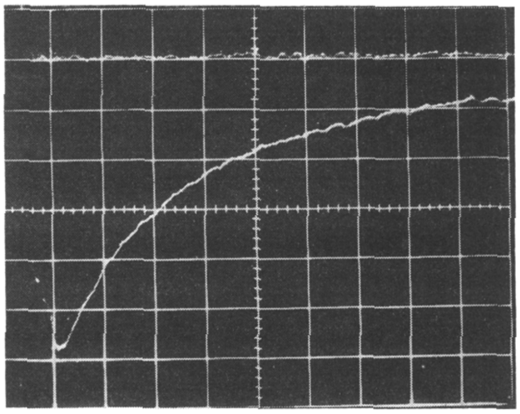


FIGURE 1: Determination of the rate of dissociation of LADH–NADH complex in the presence of 50 mM NaCl. Syringe 1, 55 μN LADH and 40 μN NADH; syringe 2, 20 mM NAD$^+$ and 100 mM NaCl. Vertical scale, 1.25% T/box; horizontal scale, 50 msec/box. Cuvet path length, 1.0 cm; wavelength, 340 mμ.

Materials and Methods

Crystalline liver alcohol dehydrogenase was prepared from horse liver by the method of Theorell *et al.* (1966). The enzyme concentration was determined by the assay method of Dalziel (1957) based on E_{280} of 0.455 ml mg^{-1} cm^{-1} for pure enzyme and by titration with NADH in the presence of isobutyramide (Theorell and McKinley-McKee, 1961a). Both methods gave comparable results. Coenzymes used were purchased either from Boehringer Corp. or Sigma Chemcial Corp. Deuterioethanol (d_6) was obtained from International Chemical and Nuclear Corporation, Irvine, Calif. Deuterated coenzyme, NADD, was prepared enzymatically from deuterioethanol by the method of Rafter and Colowick (1957).

The dissociation constant of the binary LADH–NADH complex was determined by fluorescence quenching in an apparatus designed by Dr. J. J. Holbrook similar to that described by McMurray and Trentham (1969). Stopped-flow spectrophotometric studies were performed with an instrument similar to that described by Barman and Gutfreund (1966) and with a Durrum-Gibson instrument. Relaxation experiments were carried out using an instrument designed by Dr. L. C. de Maeyer and illustrated in a recent review (Eigen, 1968). The maximum velocity was determined using an Eppendorf photometer adapted for fluorimetry with Hg 313/366 and Hg 400/3000 primary and secondary filters, respectively, to determine initial velocities. All kinetic studies were carried out at room temperature (about 23°) in 0.1 ionic strength phosphate (pH 7.0), and with addition of 50 mM NaCl where indicated.

Results

It has been reported (Theorell *et al.*, 1955) that sodium chloride caused an increased maximum velocity of the LADH reaction with ethanol and NAD$^+$ as substrates. Since the rate-limiting step under maximum velocity conditions is the dissociation rate of E$^{\text{NADH}}$ complex, this rate was determined directly by displacement with NAD$^+$ in the presence and absence of NaCl. A typical rate curve is shown in Figure 1, in which the first-order rate constant in the presence of NaCl, k_{off}, calculated from it was 9.0 sec^{-1}. The value in the absence of NaCl was 3.1 sec^{-1}. The dissociation constant of the E$^{\text{NADH}}$ complex was determined by fluorimetric titration to be 0.36 μM without NaCl and 1.05 μM in the presence of 50 mM NaCl. To ascertain that this was not due to a large change in the rate of binding of NADH to LADH, k_{on}, this value was determined by stopped-flow fluorimetry. The value for the control reaction was 1.7 × 10^7 M^{-1} sec^{-1} while in the presence of NaCl it was slightly higher, 2.5 × 10^7 M^{-1} sec^{-1}. The steady-state maximum velocity was determined fluorimetrically by studying the reaction of the enzyme with 1.1 mM NAD and a range of ethanol from 1.7 to 8.3 mM, plotting the results by the method of Lineweaver and Burk. The values obtained for V_{max} per equivalent of enzyme were 3.3 sec^{-1} in the absence of NaCl and 5.1 sec^{-1} in the presence of 50 mM NaCl. The results of these investigations are summarized in Table I. More accurate rate and binding constants will be required to ascertain whether the discrepancies between kinetically and thermodynamically determined dissociation constants are real. If they are found to be real this could be due to a two-step process during NADH binding.

TABLE I: The Effect of 50 mM NaCl on NADH Binding Rates, Dissociation Constants, and Maximum Velocity.

	$K_{\text{E,R}}$ (μM)	k_{on} (M^{-1} sec^{-1})	k_{off} (sec^{-1})	V_{max}/e (sec^{-1})
Control	0.36	1.7 × 10^7	2.8–3.2	3.3
Control plus 50 mM NaCl	1.05	2.5 × 10^7	8.5–9.5	5.1

The rate of formation of LADH-bound NADH was studied by adding saturating concentrations of NAD$^+$ and ethanol to the enzyme. A study of the dependence of the burst rate on ethanol concentration indicated that the reaction was dependent on ethanol up to a concentration of 50 mM, beyond which no further increase in rate was observed. Figure 2 illustrates the results of a typical experiment. Two distinct processes are evident: the rapid formation of 1 mole of bound NADH/equiv of enzyme sites with a rate constant of 130 sec^{-1} and a subsequent slow reaction which should be equivalent to the maximum velocity. Under the conditions of this experiment, however, the slow process was considerably inhibited due to E$^{\text{NADH}}_{\text{alcohol}}$ abortive complex formation and because the NADH produced is very tightly bound and therefore prevents further turnover of the enzyme. The reaction was studied in the presence of 50 mM NaCl resulting in a rate constant of 125 sec^{-1} for the burst rate. When d_6-deuterioethanol was used as substrate the reaction at saturation (50 mM) was much slower, with a rate constant of 25 sec^{-1}.

Since LADH binds NADH very tightly at pH 7.0 with a dissociation constant of approximately 0.3 μM it is possible to study a partial reaction, the oxidation of LADH–NADH complex by acetaldehyde. In a recent study (Bernhard *et al.*, 1970) using aromatic aldehydes, it was proposed that the two LADH–NADH–aldehyde sites per molecule of LADH were nonequivalent. To investigate this phenomenon the E$^{\text{NADH}}$ plus acetaldehyde reaction was studied with E$^{\text{NADH}}$ made by using a slight excess of LADH and E$^{\text{NADH}}$ made with a large

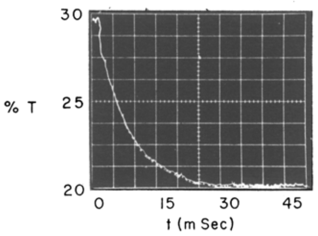


FIGURE 2: The rate of the burst of bound NADH production. Syringe 1, 4.2 mM NAD$^+$ and 100 mM ethanol; syringe 2, 44 μN LADH. Cuvet path length, 2.0 cm; wavelength, 325 mμ.

excess of the enzyme. Both complexes, when reacted with 100 μM acetaldehyde, resulted in homogeneous first-order reactions with a rate constant of 24 sec^{-1}. The total optical density change was equal to the concentration of E^{NADH} complex multiplied by the extinction coefficient. The first-order plots are shown in Figure 3.

The reaction of E^{NADH} with acetaldehyde was studied at various aldehyde concentrations and the lower curve of Figure 4 is a double-reciprocal plot of these data. It can be seen that the curve intercepts the origin, indicating that k, the rate constant for the interconversion $E^{NADH}_{aldehyde} \rightarrow E^{NAD^+}_{EtOH}$, is larger than 200 sec^{-1}. The upper curve of Figure 4 shows the results obtained using deuterated reduced coenzyme, NADD, to make the ER complex. The interconversion step is still too rapid for an intercept to be obtained even though k is smaller due to the isotope effect.

Preliminary temperature-jump relaxation studies were performed to see whether further steps in the mechanism could be characterized. The equilibrium mixture was prepared by mixing 10 mM ethanol, 1 mM NAD, and 20 μN LADH, and the perturbation of the overall reaction equilibrium was observed. A typical experimental trace is shown in Figure 5, in which two relaxation times can be seen. These are 0.22 and 0.036 sec. Sodium chloride at the 50 mM level did not change the rapid relaxation time but increased the slower time from 0.22 to 0.14 sec. If deuterioethanol was used to prepare the equilibrium mixture, the relaxation times were not significantly affected.

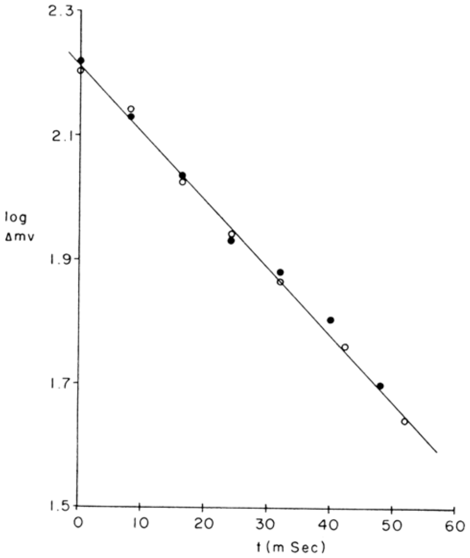


FIGURE 3: First-order plots of the reaction of LADH–NADH complex with acetaldehyde, measured at 330 mμ. The termination of the reaction, 100% T, was 1.2 V, and the cuvet path length was 1.0 cm. (○) Syringe 1, 20 μM NADH and 22 μN LADH; syringe 2, 200 μM acetaldehyde. (●) Syringe 1, 20 μM NADH and 70 μN LADH; syringe 2, 200 μM acetaldehyde.

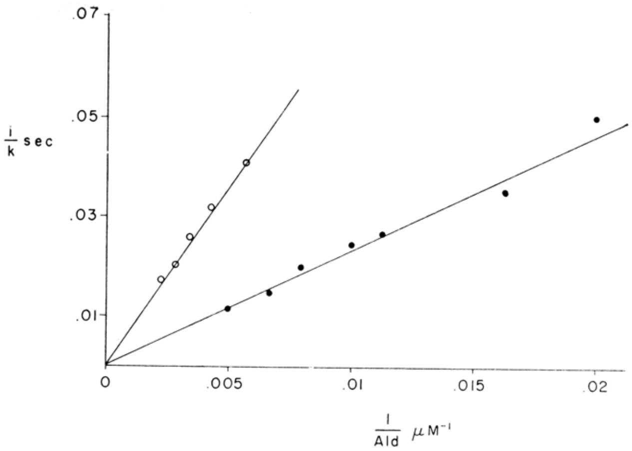


FIGURE 4: Double-reciprocal plot of observed first-order rate constants for the oxidation of LADH–coenzyme complex by acetaldehyde. (●) 6 μN LADH and 5 μM NADH after mixing; (○) 6 μN LADH and 5 μM NADD after mixing. The loss in absorbance at 325 mμ was followed.

Discussion

The data presented in Table I show a considerable difference between the maximum velocity and k_{off} for NADH in the presence of NaCl. They indicate that in the presence of NaCl the dissociation of E^{NADH} complex is not distinctly rate limiting. Another step with a rate constant of approximately 11 sec^{-1} must exist to provide the maximum velocity obtained. This result would be compatible with Scheme I, in which the isomerization would be either step 5 or 11. It is proposed that

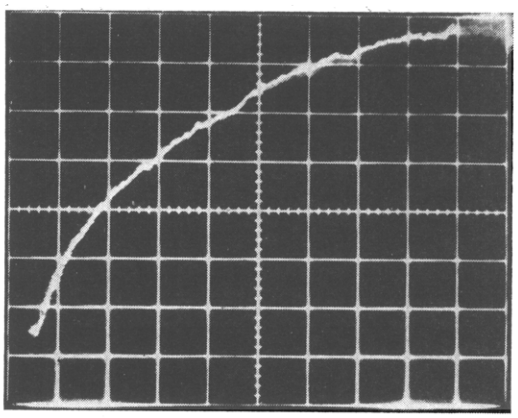
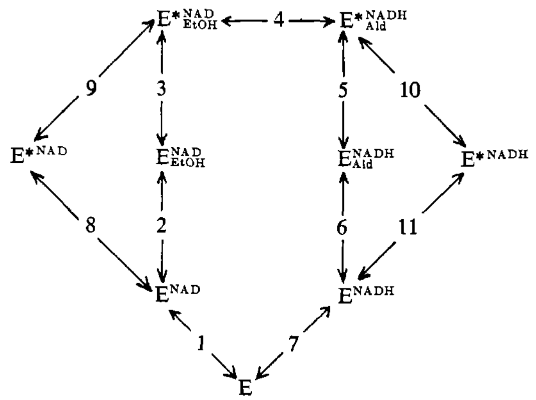


FIGURE 5: Relaxation reaction of LADH in a temperature-jump apparatus. Starting temperature was 0°, and a 30-kV discharge was used to increase temperature by 4.5°. Vertical scale, 1 mV/box; Horizontal scale, 50 msec/box. LADH concentration was 20 μN, to which 1 mM NAD$^+$ and 10 mM ethanol were added. The wavelength was 340 mμ.

SCHEME I



two steps of comparable rate, steps 7 and 5 or 11, determine the velocity of dissociation of NADH in the presence of NaCl.

The validity of using NAD^+ displacement to determine the k_{off} for NADH can be ascertained by a comparison of the fluorimetrically determined $K_{E,R}$ values and the $k_{off}:k_{on}$ ratios in Table I. In a previous study (Shore, 1969), *o*-phenanthroline was used to displace NADH and its analogs. However, the relatively slow rate of *o*-phenanthroline binding can cause errors, particularly under conditions when the coenzyme dissociation rate is rapid. The true dissociation rate of acetylpyridine coenzyme from LADH at 8° is 65 sec^{-1} when measured using NAD^+ displacement as opposed to 35 sec^{-1} reported when *o*-phenanthroline was the displacing agent.

In the study of the transient approach to the steady state, a rapid reaction with a rate constant of 130 sec^{-1}, unchanged by 50 mM NaCl, was observed. This reaction is much too rapid to be identical with the 11-sec^{-1} process elucidated by the difference between the maximum velocity and NADH dissociation rate. The high concentration of ethanol required for saturation as opposed to the lower concentrations required for saturation under steady-state conditions (Dalziel, 1963) is a reflection of the fact that under the conditions of our experiment the rate of formation of enzyme-bound NADH is being measured while in steady-state experiments the rate of dissociation of NADH from the enzyme becomes rate limiting. The substantial isotope effect observed when d_6-deuterioethanol was used as substrate indicates that the step being observed is either the hydride transfer or is controlled by the hydride-transfer rate. The magnitude of the isotope effect observed is too large to be accounted for by secondary isotope effects.

The partial reaction between E^{NADH} and aldehyde was studied for two reasons: to ascertain that the enzyme active centers are equivalent and to determine whether the dissociation rate of acetaldehyde from the ternary complex could account for either the 11-sec^{-1} step or the 130-sec^{-1} step isolated in our other experiments. The homogeneous first-order reaction for the oxidation of E^{NADH} complex by acetaldehyde, with the same rate constant regardless of whether all the active centers were saturated with NADH or only a fraction of them were complexed with coenzyme, provides evidence that under the conditions of our experiments all LADH sites were equivalent.

The reaction between E^{NADH} and varying concentrations of acetaldehyde was studied to obtain estimates of the rate constants involved. It is possible to derive a relationship for the following reaction, using the symbols R for NADH, O for NAD^+, and A for acetaldehyde

$$\mathrm{ER} + \mathrm{A} \underset{k_r}{\overset{k_f}{\rightleftharpoons}} \mathrm{ERA} \xrightarrow{k} \mathrm{EO\ alcohol}$$

At aldehyde concentrations substantially higher than ER, a pseudo-first-order reaction will be obtained. Assuming that the absorbancy of bound R is lost in the step with the rate constant k, and that the concentration of ERA is very low

$$\frac{\mathrm{d(ERA)}}{\mathrm{d}t} = 0 = k_f(\mathrm{ER} - \mathrm{ERA})(\mathrm{A}) - k_r(\mathrm{ERA}) - k(\mathrm{ERA})$$

Solving for (ERA)

$$(\mathrm{ERA}) = \frac{k_f(\mathrm{ER})}{\frac{k_r + k}{(\mathrm{A})} + k_f}$$

$$\frac{\mathrm{d(EO\ alcohol)}}{\mathrm{d}t} = k(\mathrm{ERA}) = \frac{-\mathrm{d(ER)}}{\mathrm{d}t} = \frac{kk_f(\mathrm{ER})}{\frac{k_r + k}{(\mathrm{A})} + k_f}$$

Integrating, a value for the observed first-order rate constant for the disappearance of ER is obtained

$$k_{obsd} = \frac{k}{\frac{k_r + k}{k_f(\mathrm{A})} + 1}$$

In reciprocal form

$$\frac{1}{k_{obsd}} = \frac{1}{k} + \frac{k_r + k}{k_f k}\frac{1}{(\mathrm{A})}$$

This equation indicates that the intercept of a double-reciprocal plot should provide $1/k$, the reciprocal of the hydride-transfer rate. If k is very fast compared to the range of observations, the plot will intercept the origin. Furthermore, if k is much larger than k_r, the slope will reduce to $1/k_f$, the reciprocal of the association rate of aldehyde and ER. In this case, any change in k would not affect the slope. The results of these experiments indicated that k was larger than 200 sec^{-1}, even when NADD was coenzyme. If the slope is $1/k_f$, no difference would be observed when deuterated coenzyme was used. Since the slope was considerably larger when NADD was the coenzyme, it is possible to conclude that k is not very much greater than k_r, indicating that the rate of dissociation of aldehyde from the $E^{NADH}_{aldehyde}$ complex is also quite rapid.

The data presented in this paper do not make it possible to define all the intermediate steps in the reaction sequence. However, it is reasonable to rule out certain possibilities. Since saturating concentrations of NAD^+ and ethanol were used to determine V_{max}, the 11-sec^{-1} step which becomes apparent in the presence of NaCl must be after the formation of $E^{NAD^+}_{ethanol}$ complex. The rate of formation of enzyme-bound NADH did not indicate the existence of a step as slow as 11 sec^{-1} in the burst period. This indicates that the step must be after the hydride-transfer reaction. Furthermore, the aldehyde "off" velocity is too rapid to be responsible for this transient. Consequently, the most probable explanation would be that the 11-sec^{-1} step represents an isomerization of enzyme complexes containing NADH.

The relaxation studies performed are essentially very preliminary. The two processes observed indicate a slow reaction, corresponding to the maximum velocity, and a faster process corresponding to an interconversion of enzyme complexes. The presence of NaCl did not significantly affect the rapid reaction, indicating that this process is not identical with the 11-sec^{-1} step. The preliminary nature of the relaxation study does not provide enough data to determine whether the rapid process is identical with the 130-sec^{-1} step observed in the transient approach to the steady state. Detailed studies of the concentration dependence of all the reactants have to be performed for identification of particular relaxation times with specific chemical steps.

The results of this investigation provide an indication of the type of information which may be obtained from preliminary studies of transient intermediates. This initial investigation has demonstrated the existence of an isomerization of LADH–NADH binary complex and has resulted in isolation of the hydride-transfer step. Further experiments with observations of pH and concentration dependency, as well as studies with coenzyme and substrate analogs, should provide a detailed elucidation of transients in the LADH mechanism.

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References

Barman, T. E., and Gutfreund, H. (1966), *Biochem. J. 101*, 411.

Bernhard, S. A., Dunn, M. F., Luisi, P. L., and Schack, P. (1970), *Biochemistry 9*, 185.

Criddle, R. S., McMurray, C. H., and Gutfreund, H. (1968), *Nature* (*London*) *220*, 1091.

Dalziel, K. (1957), *Acta Chem. Scand. 11*, 397.

Dalziel, K. (1963), *J. Biol. Chem. 238*, 2850.

Eigen, M. (1968), *Quart. Rev. Biophys. 1*, 3.

Heck, H. d'A., McMurray, C. H., and Gutfreund, H. (1968), *Biochem. J. 108*, 793.

Iwatsubo, M., and Pantaloni, D. (1967), *Bull. Soc. Chim. Biol. 49*, 1563.

McMurray, C. H., and Trentham, D. (1969), *Biochem. J. 115*, 913.

Rafter, G. W., and Colowick, S. P. (1957), *Methods Enzymol. 3*, 887.

Shore, J. D. (1969), *Biochemistry 8*, 1588.

Shore, J. D., and Theorell, H. (1966), *Arch. Biochem. Biophys. 116*, 255.

Theorell, H., and Chance, B. (1951), *Acta Chem. Scand. 5*, 1127.

Theorell, H., and McKinley-McKee, J. S. (1961a), *Acta Chem. Scand. 15*, 1797.

Theorell, H., and McKinley-McKee, J. S. (1961b), *Acta Chem. Scand. 15*, 1811.

Theorell, H., Nygaard, A. P., and Bonnichsen, R. (1955), *Acta Chem. Scand. 9*, 1148.

Theorell, H., Taniguchi, S., Åkeson, Å., and Skursky, L. (1966), *Biochem. Biophys. Res. Commun. 24*, 603.

Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.