Aaronson, R. P., and Frieden, C. (1972), *J. Biol. Chem. 247,* 7502.
Alpers, J. B., Paulus, H., and Bazylewicz, G. A. (1971), *Proc. Nat. Acad. Sci. U. S. 68,* 2937.
Bates, D. J., and Frieden, C. (1973), *Comput. Biomed. Res. 6,* 474.
Bock, P., and Frieden, C. (1974), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 33,* 1413.
Frieden, C. (1968), *in* The Regulation of Enzyme Activity and Allosteric Interactions, Kvamme, E., and Phil, A., Ed., New York, N. Y., Academic Press, p 59.
Frieden, C. (1971), *Annu. Rev. Biochem. 40,* 653.
Hofer, H. W. (1971), *Hoppe-Seyler's Z. Physiol. Chem. 352,* 997.
Hofer, H. W., and Pette, D. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349,* 1105.
Hulme, E. C., and Tipton, K. F. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 12,* 197.
Kono, N., and Uyeda, K. (1973), *J. Biol. Chem. 248,* 8603.
Lad, P. M., Hill, D. E., and Hammes, G. G. (1973), *Biochemistry 12,* 4303.
Leonard, K. R., and Walker, I. O. (1972), *Eur. J. Biochem. 26,* 442.
Ling, K. H., Marcus, F., and Lardy, H. A. (1965), *J. Biol. Chem. 240,* 1893.
Mansour, T. E. (1965), *J. Biol. Chem. 240,* 2165.
Paetkau, V., and Lardy, H. A. (1967), *J. Biol. Chem. 242,* 2035.
Parmeggiani, A., Luft, J. H., Love, D. S., and Krebs, E. G. (1966), *J. Biol. Chem. 241,* 4625.
Pavelich, M. J., and Hammes, G. G. (1973), *Biochemistry 12,* 1408.

The Interaction of Cyclic 3′,5′-Adenosine Monophosphate with Yeast Glyceraldehyde-3-phosphate Dehydrogenase. I. Equilibrium Dialysis Studies†

Judi Milne and Robert A. Cook*

ABSTRACT: Binding studies with yeast glyceraldehyde-3-phosphate dehydrogenase indicate a maximum of four binding sites for the inhibitor cyclic 3′,5′-AMP and three binding sites for the substrate NAD^+. The binding data for cAMP indicate simple noncooperative binding with the binding affinity inversely related to temperature. The binding data for NAD^+, however, indicate positive and negative cooperative behavior, consistent with a ligand induced sequential model. Competition experiments indicate that NAD^+ inhibits the binding of cAMP in a noncompetitive manner. Similarly cAMP inhibits the binding of NAD^+ noncompetitively. The results indicate that cAMP and NAD^+ do not compete for the same binding site on the enzyme surface. The suggestion is made that cAMP inhibits enzyme activity by binding to a specific regulatory site rather than competing for the NAD^+ binding site.

Glyceraldehyde-3-phosphate dehydrogenase of yeast has been the subject of numerous physicochemical and kinetic studies due to the availability of large quantities of homogeneous enzyme and the central role this enzyme has played in the development of allosteric theory. The enzyme is generally accepted to be tetrameric in structure, composed of four apparently identical monomers (Harris and Perham, 1965). The binding of NAD^+ to the enzyme has been shown to be cooperative in nature by various techniques at several different temperatures (Cook and Koshland, 1970; Kirschner *et al.*, 1971; Ellenrieder *et al.*, 1972; Sloan and Velick, 1973). We have suggested, however, that the overall shape of the binding curves indicates greater complexity (Cook and Koshland, 1970). Specifically, distinct breaks occurred in the Hill plots at approximately 50% saturation and the Scatchard plots did not extrapolate to four which was expected on the basis of the number of subunits. The anomalies were explained by a ligand-induced sequential model (Koshland *et al.*, 1966; Kirtley and Koshland, 1967) with subunit interactions which led to a mixture of positive and negative cooperativity. Initial velocity kinetic studies with the yeast enzyme were consistent with this explanation (Koshland *et al.*, 1970). Qualitatively, similar subunit interactions resulting in mixed positive and negative cooperativity due to a single ligand binding have been observed with aspartate transcarbamylase (Cook, 1972).

The observation that cyclic 3′,5′-AMP was a potent inhibitor of the yeast enzyme (Yang and Deal, 1969) raised some interesting questions regarding the effect this inhibitor would have on the binding and kinetic behavior of the enzyme. This paper reports the stoichiometry of cAMP[1] binding to the yeast enzyme and some ligand binding interactions, as measured by equilibrium dialysis. The effect of cAMP on initial velocity kinetic studies of the enzyme are presented in the following paper (Rock and Cook, 1974).

Experimental Section

Materials. Glyceraldehyde-3-phosphate dehydrogenase (GPD) was purified from Red Star brand baker's yeast following the procedure of Krebs (1955). After four crystalliza-

† From the Department of Biochemistry, University of Western Ontario, London, Ontario, Canada. *Received November 28, 1973.* This work was supported by a research grant from the Medical Research Council of Canada (MA-3848).

[1] Abbreviations used are: GPD, glyceraldehyde-3-phosphate dehydrogenase; cAMP, cyclic 3′,5′-adenosine monophosphate; n_H, Hill coefficient; n_m, maximum number of binding sites.

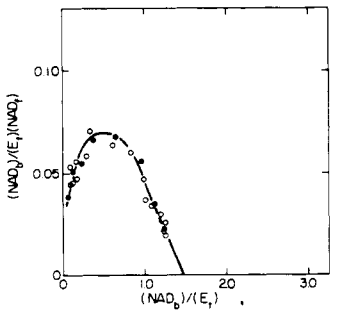

FIGURE 1: Scatchard plot of binding of NAD^+ to "native" yeast GPD at 4°. Open and closed circles represent two individual experiments at different enzyme concentrations. Conditions: 0.05 M sodium pyrophosphate buffer, 1 mM EDTA (pH 8.5), 4°, and (●) 9.54×10^{-5} M GPD, (○) 17.14×10^{-5} M GPD.

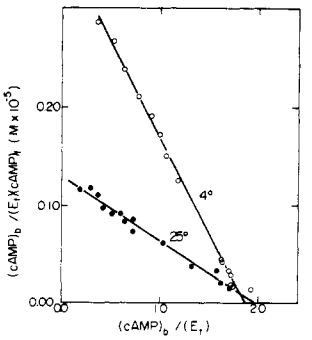

FIGURE 3: Scatchard plot of binding of cAMP to "native" yeast GPD at 4 and 25°. Conditions: 0.05 M sodium pyrophosphate buffer–1 mM EDTA (pH 8.5), 9.54×10^{-5} M GPD at 4° (○), 12.0×10^{-5} M GPD at 25° (●).

tions, the enzyme appeared to be homogeneous when tested by cellogel electrophoresis in Tris-borate buffer (pH 8.6) (Boyer *et al.*, 1963) or by disc electrophoresis (Ornstein and Davis, 1964). Prior to equilibrium dialysis studies, crystals of the enzyme were centrifuged down, dissolved in and dialyzed against 0.05 M sodium pyrophosphate buffer (pH 8.5), containing 1 mM EDTA and 0.1 mM dithiothreitol. Protein concentration was determined spectrophotometrically at 280 mμ using a molar extinction coefficient determined by Krebs (1955) of 1.35×10^5 (corrected for a molecular weight of 145,000). Protein concentrations were also estimated by the method of Lowry *et al.* (1951) with bovine serum albumin as reference standard, and gave essentially identical results. In all experiments reported here, an enzyme preparation with a specific activity 220,000–250,000 was used. (One unit is defined as an increase of 0.001 at 340 mμ per min per mg of protein.) The enzyme was found to be stable for no longer than 3 months when stored as the crystalline suspension in ammonium sulfate containing EDTA (1 mM) and dithiothreitol (1 mM).

After four crystallizations, the enzyme exhibited an OD 280:260 ratio of 1.5–1.6. Such a ratio would correspond to approximately 1.5 mol of NAD^+ bound to the native enzyme as estimated by the method of Fox and Dandliker (1956) or Kirschner *et al.* (1971). However, heat precipitation followed by a fluorometric determination of the released NAD^+ (Lowry *et al.*, 1957) indicated essentially no NAD^+ bound to the native enzyme.

The OD_{260} absorbing material was removed from the enzyme by charcoal-Celite treatment according to the method of Murdock and Koeppe (1964). The resultant enzyme exhibited an OD 280:260 ratio of 2.1. The nature of the bound material has not been determined. The specific activity of the enzyme was not significantly altered by charcoal treatment. Equilibrium dialysis experiments were performed immediately following charcoal treatment of the enzyme.

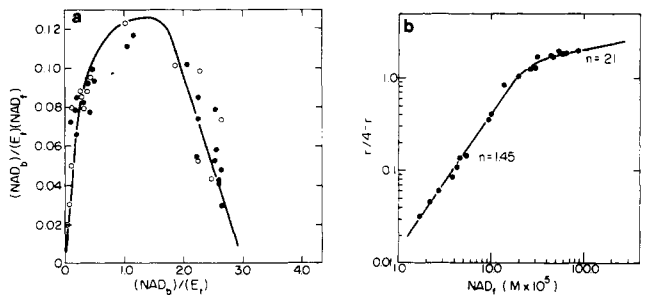

FIGURE 2: Equilibrium dialysis study of binding of NAD^+ to charcoal-treated yeast GPD at 4°. Open and closed circles represent two individual experiments at different enzyme concentrations. Conditions: 0.05 M sodium pyrophosphate buffer, 1 mM EDTA (pH 8.5), 4°, and (●) 8.79×10^{-5} M GPD, (○) 10.54×10^{-5} M GPD. (a) Scatchard plot; (b) Hill plot.

Equilibrium dialysis was routinely carried out in 0.15-ml cells using Visking 20/32 dialysis tubing. The dialysis tubing was washed several times in hot EDTA and then pyrophosphate buffer (pH 8.5), but was not otherwise treated to increase porosity. Controls of NAD^+ *vs.* buffer indicated that equilibrium was reached in 15 hr at 25° and 20 hr at 4°. Controls of cAMP *vs.* buffer indicated that equilibrium was reached in 10 hr at 25 and 37° and 20 hr at 4°. The presence of enzyme did not affect the time of equilibration. The enzyme was checked periodically for any denaturation during experiments by using a standard reaction mixture which contained 50 μmol of sodium pyrophosphate (pH 8.5), 10 μmol of sodium arsenate, 0.468 μmol of glyceraldehyde 3-phosphate, and 0.650 μmol of NAD^+ in a 1.2 ml total volume. After equilibrium was reached, ligand concentration was determined on aliquots from each cell compartment. Samples of 0.075 ml were counted in 10 ml of Bray's (1960) solution in a scintillation counter and monitored for possible quenching. The counts were not held constant but varied from 2,000 to 200,000 cpm. All samples were therefore counted to constant efficiency.

$[^{14}C]NAD^+$ with a specific activity of 59 Ci/mol was purchased from Amersham. Cyclic 3′,5′-$[^3H]$AMP with a specific activity of 16.3 Ci/mmol was purchased from Schwartz Bioresearch. NAD^+ was purchased from Boehringer und Sohne, Mannheim. Glyceraldehyde 3-phosphate diethylacetal barium salt was purchased from Sigma and converted into the free acid as described by Sigma.

Results

Binding Studies of NAD^+ to Yeast GPD. In an earlier study (Cook and Koshland, 1970), it was shown by equilibrium dialysis studies that the binding of NAD^+ to the yeast enzyme was optimal at 4°. The results of that study were explained on the basis of a mixture of positive and negative cooperative behavior in the binding of NAD^+ to the enzyme. In the present study, essentially identical results were obtained from the NAD^+ binding studies.

Equilibrium dialysis with yeast GPD was carried out as previously described in the Experimental Section. The binding curve for NAD^+ at 4° to the "native" enzyme exhibits sigmoidicity at low NAD^+ concentrations. When the data were replotted in the form of the Scatchard plot, a curve characteristic of positive cooperativity was observed (Figure 1), but the extrapolated n_m value was only 1.5. The native enzyme exhibited an OD 280:260 ratio of 1.5, as previously described in the Ex-

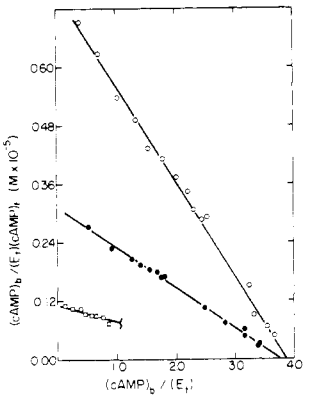

FIGURE 4: Scatchard plot of binding of cAMP to charcoal-treated yeast GPD at 4, 25, and 37°. Conditions: 0.05 M sodium pyrophosphate buffer–1 mM EDTA (pH 8.5), 5.14×10^{-5} M GPD at 4° (○), 25° (●), and 37° (■).

perimental Section. Following charcoal treatment of the enzyme, the OD 280:260 ratio was raised 2.1. The binding curve for NAD^+ at 4° with the charcoal-treated enzyme exhibits obvious cooperativity at low NAD^+ concentrations. When the data were replotted in the Scatchard plot, a curve characteristic of positive cooperativity was observed (Figure 2a), but the extrapolated n_m value was now 3.0. Plotting the data of Figure 2a in a Hill plot gives n_H values of 1.45 below 50% saturation and 0.21 above 50% saturation (Figure 2b). The results presented in Figure 2 with the charcoal-treated enzyme are analogous to the results previously published with untreated enzyme (Cook and Koshland, 1970). Charcoal treatment did not appear to affect the association constant for NAD^+ (approximately 1×10^4 M) as determined from the linear portions of the Scatchard plots (Figures 1 and 2a), but did increase the total number of binding sites for NAD^+.

Binding Studies of cAMP to Yeast GPD. A typical Scatchard plot for the binding of cAMP to the "native" enzyme at 4 and 25° is presented in Figure 3. The binding of cAMP is linear, indicating no cooperativity with an extrapolated n_m value of approximately 2. The binding of cAMP is approximately three times stronger at 4° ($K_{av} = 1.9 \times 10^4$) than at 25° ($K_{av} = 0.66 \times 10^4$ M).

Binding curves for cAMP with the charcoal-treated enzyme at 4, 25, and 37° indicated no cooperative behavior at any temperature. At 37°, concentrations of cAMP greater than 0.15 mM caused precipitation of the enzyme in the dialysis cell. When the data were replotted in the Scatchard plot (Figure 4), linear lines were again observed with extrapolated n_m values of approximately 3.7–3.9. The strength of binding of cAMP to the enzyme appeared to be inversely related to temperature, analogous to the binding of NAD^+. The average association constants determined from the slopes of the lines in Figure 4 were 1.96×10^4 M at 4°, 0.84×10^4 M at 25°, and 0.36×10^4 M at 37°. Charcoal treatment of the enzyme did not alter significantly the affinity of the enzyme for cAMP, but did increase the total number of binding sites (see Figures 3 and 4).

The effect of increasing NAD^+ concentrations on the binding of cAMP to the charcoal-treated enzyme at 4° was tested. The binding of cAMP is obviously inhibited by the presence of NAD^+. When the data are replotted in the Klotz plot (Figure 5a), noncompetitive inhibition of cAMP binding by NAD^+ is observed. When the results are presented in a Scatchard plot (Figure 5b), it is obvious that increasing NAD^+ concentrations progressively decrease the binding affinity for cAMP on the enzyme and decrease the total number of moles of cAMP bound/mole of enzyme at saturation. Similar results were obtained in the reciprocal experiment when NAD^+ binding was determined in the presence of increasing cAMP concentration.

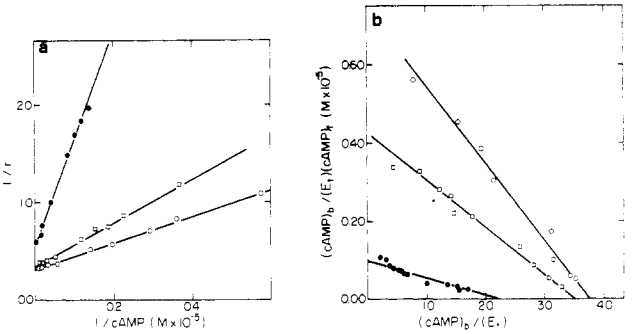

FIGURE 5: Equilibrium dialysis study of binding of cAMP to charcoal-treated yeast GPD at 4° in the presence of NAD^+. Conditions: 0.05 M sodium pyrophosphate buffer–1 mM EDTA (pH 8.5), 4°, 6.76×10^{-5} M GPD. Control (no NAD^+) (○), 81.8×10^{-5} M NAD (□), 136×10^{-5} M NAD (●). (a) Klotz plot; (b) Scatchard plot.

Experiments attempted with different enzyme preparations and at several GPD concentrations in the region of 5–25 mg/ml gave similar results to those reported here. There was no loss in enzyme activity during the duration of the equilibrium dialysis experiments as indicated by recovery of enzyme activity after the dialysis procedure was completed. Sedimentation velocity experiments indicated that the $s_{20,w}$ value of the enzyme was unaltered by the presence of saturating concentrations of NAD^+ and/or cAMP.

Discussion

Glyceraldehyde-3-phosphate dehydrogenase has been isolated from Red Star brand baker's yeast by the conventional method of Krebs (1955). Surprisingly, the enzyme after four crystallizations exhibited an OD 280:260 ratio of 1.5, indicating approximately 1.5 mol of NAD bound/mol of enzyme. A more direct fluorometric determination, however, revealed no NAD^+ bound to the enzyme. The nature of the OD_{260} absorbing material on the enzyme has not been determined. In previous studies, enzyme isolated from the same brand of yeast by the same procedure exhibited an OD 280:260 ratio of 1.85 and was free of bound NAD^+. The differences observed in the present study would indicate either a change in strain or growth conditions of the commercially obtained yeast or some subtle, unknown difference in the purification procedure of the enzyme.

The enzyme, as isolated, binds approximately 1.5 mol of NAD^+/mol of enzyme with positive cooperativity (Figure 1) and approximately 2 mol of cAMP/mol of enzyme with no cooperativity (Figure 3). These results would suggest that the OD_{260} absorbing material bound to the enzyme interferes with both NAD^+ and cAMP binding. This conclusion is supported by the studies on charcoal-treated enzyme (OD 280:260 of 2.1) which binds 3.0 mol of NAD/mole of enzyme (Figure 2), and 4.0 mol of cAMP/mol of enzyme (Figure 4).

The binding of NAD^+ to the charcoal-treated enzyme is qualitatively similar to the results previously reported with untreated enzyme (Cook and Koshland, 1970). The maximum n_m value obtained at 4° is 3.0 (Figure 2a) and the Hill plot is biphasic, with a break occurring at approximately 50% saturation (Figure 2b). The two n_H values from the Hill plot of 1.45 below 50% saturation and 0.21 above 50% saturation are significantly different from the previous results, indicating weaker positive cooperativity at low NAD^+ concentrations and stronger negative cooperative behavior at high NAD^+ concentrations. The weak positive cooperative behavior is also observed

in initial velocity kinetic studies (Rock and Cook, 1974). Charcoal treatment of the enzyme does not appear to affect the binding affinity for NAD^+ as estimated roughly from the linear portion of the Scatchard plots. Similarly, the binding affinity for cAMP does not appear to be altered by charcoal treatment of the enzyme.

The binding of NAD^+ to the enzyme is consistent with the model previously proposed by Cook and Koshland (1970), *i.e.*, the binding of the first molecule of NAD^+ makes the binding of the second easier, but when the second molecule is bound, the binding of the third and fourth NAD^+ molecules is made progressively more difficult. This model has been questioned by Ellenrieder *et al.* (1972) who have examined the binding of NAD^+ and NADH to the yeast enzyme. Equilibrium dialysis studies by these authors have indicated that NAD^+ does bind with positive cooperativity but a total of 4 mol of NAD^+ were bound/mol of enzyme. The discrepancy arises due to the different assumptions about the presence or absence of inactive (and nonbinding) protein in the enzyme preparations. The binding results of Ellenrieder *et al.* (1972) have been corrected for the presence of inactive enzyme by a factor determined from measurng the specific activity of the enzyme and the absorption of the saturated enzyme–NAD^+ complex (Kirschner *et al.*, 1971; Kirschner and Voigt, 1968).

No such corrections for inactive enzyme have been applied to the present results. We have found previously that the specific activity of the enzyme was somewhat dependent on the ionic conditions prior to or during assay. The precise specific activity therefore could not be taken as an index of enzyme purity in our hands. In addition, the suggestion that inactive protein must also be nonbinding is a tenuous conclusion. The results of the cAMP binding studies are significant in this regard. Approximately 4 mol of cAMP were bound/mol of enzyme at 4 and 25°, a number consistent with the proposed subunit structure, while only 3 mol of NAD^+ were bound under similar conditions. If the reason for the low NAD^+ binding is due to the presence of inactive, nonbinding protein, it is surprising that cAMP binding is not also affected, unless one assumes that inactivation of enzyme only affects the NAD^+-binding sites. Although such a premise is possible, we do not consider it likely. Studies with aged enzyme preparations have shown a decrease in both NAD^+ and cAMP binding, indicating denaturation, will affect the binding of both ligands.

A more significant feature of the NAD^+-binding curves in regard to the proposed model was the failure of the binding curve to plateau at "saturation" (Cook and Koshland, 1970). The results presented in this paper are qualitatively similar. The results of Ellenrieder *et al.* (1972) apparently do not exhibit any "tailing-off" of NAD^+ binding even without correction for inactive protein. More recent binding studies by the method of fluorescence-quenching titration (Sloan and Velick, 1973) also do not exhibit any upward deflection of NAD^+ binding near saturation. The extent to which these differences are due to the analytical methods employed, differences in the yeast strains from which the enzymes were prepared, or variations in the isolation and storage of the sensitive oligomeric protein remains to be determined.

The inhibition of the activity of the yeast enzyme by cAMP has been suggested to be due to competition of cAMP for the NAD^+-binding site (Yang and Deal, 1969). The observation that charcoal treatment simultaneously increased both cAMP and NAD binding would support such a conclusion. However, when [^{3}H]cAMP binding was measured in the presence of cold NAD^+, noncompetitive inhibition was observed (Figure 5). Both the affinity and the total number of binding sites for cAMP were decreased by the presence of NAD^+. These results would indicate that NAD^+ and cAMP do not compete for the same site on the enzyme surface. Similarly, the binding of NAD^+ to the enzyme is inhibited noncompetitively by cAMP. These results raise the possibility that cAMP may bind to a regulatory site on the enzyme and inhibit the binding of NAD^+ *via* a conformational change in the enzyme. The results of initial velocity kinetic studies are consistent with this suggestion (Rock and Cook, 1974). Similarly, the presence of NAD^+ appears to affect the binding of cAMP, again presumably *via* a conformational change in the enzyme.

The binding of cAMP is approximately three times stronger than the binding of NAD^+ to the enzyme, as would be expected for an allosteric effector. The binding of cAMP does not appear to cause any interaction between subunits as indicated by the linear Scatchard plots, which indirectly supports the argument that NAD^+ and cAMP do not bind to the same site. The results presented here cannot prove, however, that cAMP has a unique regulatory site on the enzyme surface. A more direct proof of this suggestion would be to desensitize the enzyme to the effect of cAMP by a method such as photooxidation treatment while maintaining enzyme activity. Such an approach has been used successively in studies of the cAMP effect on human phosphofructokinase (Lee *et al*, 1973). Such studies are presently under investigation in this laboratory.

References

Boyer, S. H., Fainer, D. C., and Naughton, M. A. (1963), *Science 140,* 1228.

Bray, G. A. (1960), *Anal. Biochem. 1,* 279.

Cook, R. A. (1972), *Biochemistry 11,* 3792.

Cook, R. A., and Koshland, D. E., Jr. (1970), *Biochemistry 9,* 3337.

Ellenrieder, G. V., Kirschner, K., and Schuster, I. (1972), *Eur. J. Biochem. 26,* 220.

Fox, J. B., Jr., and Dandliker, W. B. (1956), *J. Biol. Chem. 218,* 53.

Harris, J. I., and Perham, R. N. (1965), *J. Mol. Biol. 13,* 876.

Kirschner, K., Gallego, E., Scuster, I., and Goodall, D. (1971), *J. Mol. Biol. 58,* 29.

Kirschner, K., and Voigt, B. (1968), *Hoppe-Seyler's Z. Physiol. Chem. 349,* 632.

Kirtley, M. E., and Koshland, D. E., Jr., (1967), *J. Biol. Chem. 242,* 4192.

Koshland, D. E., Jr., Cornish-Bowden, A. J., and Cook, R. A. (1970), *in* Pyridine-Nucleotide-Dependent Dehydrogenases, Sund, H., Ed., West Berlin, Springer-Verlag, p 199.

Koshland, D. E., Jr., Nemethy, G., and Filmer, D. (1966), *Biochemistry 5,* 365.

Krebs, E. G. (1955), *Methods Enzymol. 1,* 407.

Lee, L. M. Y., Krupka, R. A., and Cook, R. A. (1973), *Biochemistry 12,* 3503.

Lowry, O. H., Robert, N. R., and Kapphahn, J. I. (1957), *J. Biol. Chem. 224,* 1047.

Lowry, O. H., Rosebrough, J. N., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193,* 265.

Murdock, R., and Koeppe, O. J. (1964), *J. Biol. Chem. 239,* 1983.

Ornstein, L., and Davis, B. J. (1964), *Ann. N.Y. Acad. Sci. 121,* 321.

Rock, M. G., and Cook, R. A. (1974), *Biochemistry 13,* 4200.

Sloan, D. L., and Velick, S. F. (1973), *J. Biol. Chem. 248,* 5419.

Yang, S. T., and Deal, W. C., Jr. (1969), *Biochemistry 8,* 2806.