Lagunas, R., McLean, P., and Greenbaum, A. L. (1970), *Eur. J. Biochem. 15*, 179.
Lineweaver, H., and Burk, D. (1934), *J. Amer. Chem. Soc. 56*, 658.
Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951), *J. Biol. Chem. 193*, 265.
Luzzatto, L., and Afolayan, A. (1971), *Biochemistry 10*, 420.
Reich, J. G., Till, U., Gunther, J., Zhan, D., Tschisgale, M., and Frunder, H. (1968), *Eur. J. Biochem. 6*, 384.
Rudack, D., Gozukara, E. M., Chisolm, E. M., and Holten, D. (1971), *Biochim. Biophys. Acta 252*, 305.
Shen, L. C., and Atkinson, D. E. (1970), *J. Biol. Chem. 245*, 5974.
Veech, R. L., Eggleston, L. V., and Krebs, H. A. (1969), *Biochem. J. 115*, 609.
Villet, R. H., and Dalziel, K. (1969), *Biochem. J. 115*, 639.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.
Williamson, J. R., Browning, E. T., and Scholz, R. (1969), *J. Biol. Chem. 244*, 4607.
Wratten, C. C., and Cleland, W. W. (1963), *Biochemistry 2*, 935.

Role of Zinc(II) in the Refolding of Guanidine Hydrochloride Denatured Bovine Carbonic Anhydrase†

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ABSTRACT: Circular dichroism, ultraviolet difference spectroscopy, and activity measurements (*p*-nitrophenyl acetate as substrate) have been employed to study the denaturation and renaturation of bovine carbonic anhydrase B. This metalloenzyme is readily denatured by guanidine hydrochloride and refolds spontaneously upon removal of the denaturing condition, recovering essentially full (>95%) enzymatic activity. Denaturation in 3 M guanidine hydrochloride at 25° and pH 6, as judged by circular dichroism and ultraviolet spectroscopy, carries both the native and the apoenzyme from the same initial to the same final conformational state, exposing approximately six buried tryptophan side chains, disrupting interactions of aromatic side chains with dissymmetrical regions, and presumably destroying the specific zinc binding site of the enzyme. The transition between native and denatured conformational states appears thermodynamically reversible with or without Zn(II), although in the absence of the metal it occurs at a lower guanidine hydrochloride concentration (1.5 M *vs.* 1.0 M midpoint). Renaturation kinetics are complex and imply that intermediate species accumulate during the reaction. Under some conditions (dilution from 4.0 M to 1.0 M guanidine hydrochloride, pH 6, 25°) refolding occurs readily if Zn(II) is present during the initial stages of the reaction, whereas it occurs at an extremely low rate if Zn(II) is added later. This suggests that Zn(II) is bound during the initial steps of folding of the polypeptide chain and thus influences the pathway of the reaction although it does not affect the final conformational state.

Bovine carbonic anhydrase (carbonate hydro-lyase, EC 4.2.1.1) is a metalloenzyme containing one atom of bound zinc(II) per molecule of 30,000 molecular weight. The zinc atom can be removed and replaced, as originally shown by Lindskog and Malmström (1962). Enzyme activity disappears on removal of the zinc, and returns when zinc is added to the apoenzyme (Lindskog, 1963). Thermodynamic parameters for the binding of zinc to the apoenzyme, obtained by combining the calorimetric results of Henkens *et al.* (1969) with equilibrium measurements of Lindskog and Malmström (1962), show that the zinc is very tightly bound and that an unfavorable enthalpy of binding is overwhelmed by a very large entropy increase. The binding of zinc(II) by the apoenzyme is relatively rapid while its dissociation is very slow (Henkens and Sturtevant, 1968; Henkens and Lochmüller, 1970). Zinc binding is accompanied by only very small changes in the ultraviolet spectrum (Henkens and Sturtevant, 1968) and almost no change in the ultraviolet circular dichroism. These observations, together with the observation that zinc binding has little effect on the rotational relaxation time or the sedimentation constant of the protein (Brewer *et al.*, 1968), indicate that the metal has little or no effect on the structure of the protein. The identity of the protein ligands coordinating the zinc is unknown. Presumably the zinc binding site, which in the human enzyme is located at the bottom of a crevice (Fridborg *et al.*, 1967), is destroyed on denaturation of the enzyme by guanidine hydrochloride.

Many enzymes denatured by guanidine hydrochloride have been shown to refold spontaneously to fully active enzymes, evidently native in all respects upon removal of the denaturing conditions (*cf.* review by Tanford, 1968). These experiments provide convincing evidence that the three-dimensional structure of an enzyme is determined by its primary structure, although they do not rule out the possibility that the presence or absence of specific ions or molecules may determine whether or not the native structure is achieved. In this regard, there has been no systematic study of the role of metal ions in the refolding of a metalloenzyme, although Teipel and Koshland (1971) reported some work on the metal-activated enzyme enolase and Reynolds and Schlesinger (1969) showed

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that refolding of alkaline phosphatase monomer could take place in the absence of metal. Consequently, we have undertaken studies of the role of zinc(II) in the refolding of guanidine hydrochloride denatured carbonic anhydrase. Experiments in which Zn^{2+} was added either before or after refolding of the apoenzyme demonstrate that the presence of zinc during refolding is not required for recovery of full enzymatic activity; however, under some conditions its absence during the refolding process has important kinetic consequences.

Experimental Section

Bovine erythrocyte carbonic anhydrase was purchased from Worthington. Component B was prepared by chromatography on DEAE-cellulose according to the method of Lindskog (1960), lyophilized, and stored in the cold. Protein concentrations were determined from the absorbance at 280 nm using a molar absorptivity of 5.7×10^4 M^{-1} cm^{-1} (Lindskog and Nyman, 1964). Enzymatic activity was determined with *p*-nitrophenyl acetate as substrate using the method previously reported (Henkens and Sturtevant, 1968). The active enzyme concentration determined by titration with 5-acetamido-1,3,4-thiadiazole-2-sulfonamide (acetazolamide) with *p*-nitrophenyl acetate as substrate (Kernohan, 1965) was 88% of the protein concentration determined from the absorbance at 280 nm. The zinc-free apoenzyme was prepared by dialysis against 1,10-phenanthroline in 0.1 M acetate buffer, pH 5.2 (Lindskog and Malmström, 1962), until the esterase activity was less than 3% of its original value. The apoenzyme was then dialyzed against water, lyophilized, and stored in the cold. Addition of an equivalent of $ZnCl_2$ to a solution of this apoenzyme preparation led to the recovery of full enzymatic activity (*p*-nitrophenyl acetate as substrate).

Deionized water provided by Continental Water Service was filtered through a 0.45-μm Gelman membrane and used to prepare all aqueous solutions. Acetozolamide was purchased from Koch-Light Laboratories, recrystallized in water, and dissolved in 2 equiv of NaOH. Zinc solutions were prepared by dissolving reagent grade zinc metal in a slight excess of HCl.

Absorbance measurements were made with a Cary 15 spectrophotometer. Circular dichroism measurements were made with the Durrum-Jasco J-15 in round fused silica cells. The slit width on the Durrum-Jasco instrument was programmed to give a spectral bandwidth of 2 nm. All measurements were made in unbuffered solutions (pH 6) near 25°.

Results

Denaturation Ultraviolet Difference Spectra. The ultraviolet difference spectra resulting from the denaturation of carbonic anhydrase by guanidine hydrochloride are shown in Figure 1. The observed spectral changes are characteristic of those observed for a transfer of aromatic chromophores from an environment like the interior of a protein to an aqueous environment. The difference peak at 292 nm is assigned, by analogy with the numerous studies of proteins and model compounds, to a blue shift in the $\pi \rightarrow \pi^*$ absorption band of the indole chromophore of tryptophan residues (*cf.* review by Donovan, 1969). In 3 M guanidine hydrochloride where, as judged by the difference spectra, the denaturation transition appears complete, the change in molar absorptivity, $\Delta\epsilon_{292}$, is -6.7×10^3. Comparison of this value with the results of solvent perturbation studies permits a rough estimation of the number of tryptophan side chains transferred from the

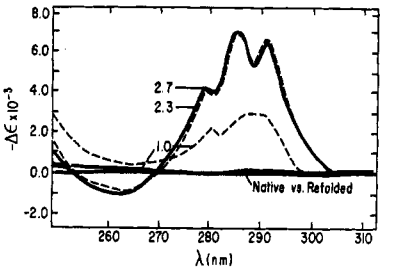

FIGURE 1: Denaturation ultraviolet difference spectra of carbonic anhydrase at pH 6, 25°. The sample cell contained the molar concentration of guanidine hydrochloride indicated by the numbers next to the curves. Both sample and reference cells contained the same concentration of carbonic anhydrase, usually *ca.* 3×10^{-5} M in an unbuffered solution. Dashed lines, apoenzyme; solid lines, holoenzyme. Spectra corrected for small absorbance due to guanidine hydrochloride.

interior of the protein into the solvent as a result of the denaturation transition. Taking the change in molar absorptivity as $\Delta\epsilon_{292} = -1600$ per indole group for the transfer from the interior of a protein into water (Donovan, 1964) and $\Delta\epsilon_{292} = 410$ per group for solvent perturbation by 3 M guanidine hydrochloride (Hamaguchi *et al.*, 1963) we calculate a value of $\Delta\epsilon_{292} = -1.2 \times 10^3$ per group for the transfer of indole from the interior of a protein into 3 M guanidine hydrochloride. Comparison of this value with the observed change in molar absorptivity on denaturation suggests that approximately six buried tryptophan side chains are exposed during the denaturation transition.

The ultraviolet difference spectrum in the aromatic region resulting from the denaturation of the zinc-free apoenzyme (Figure 1) is virtually identical with that for the zinc enzyme indicating that the same number of buried tryptophan side chains are exposed on denaturation.

Ultraviolet Circular Dichroism. The circular dichroism of bovine carbonic anhydrase B, in the region 240–310 nm, shown in Figure 2, contains a major positive dichroic absorption band near 245 nm, a negative band near 270 nm, and a number of small bands above 270 nm. It is similar to the circular dichroism of bovine carbonic anhydrase B reported by Coleman (1968). It is also very similar to the circular dichroism of human carbonic anhydrase C (Beychok *et al.*, 1966) although differences are observed. The circular dichroism of human enzyme C and bovine enzyme B are both significantly different from that of human carbonic anhydrase B, especially in the region below 260 nm.

Beychok *et al.* (1966) have discussed the origin of the Cotton effects above 240 nm in the human enzymes B and C and concluded that they must arise from asymmetrical interaction of the aromatic side chains with other regions of the enzyme. The same arguments and conclusions apply to the bovine enzyme (*cf.* review by Timasheff, 1970).

The circular dichroism of apobovine carbonic anhydrase B at both pH 5 and pH 7 is virtually identical with that of the zinc enzyme (Figure 2), indicating that the loss of zinc in this enzyme does not importantly affect the protein structure around the aromatic chromophores. This is consistent with the observation that removal of the zinc causes only small changes in the ultraviolet spectrum (Henkens and Sturtevant, 1968). In contrast, optical rotatory dispersion studies of human carbonic anhydrase B indicate that at pH 5.5 (although not at pH 7.5) removal of the zinc results in reversible changes in the structure of the enzyme (Coleman, 1965).

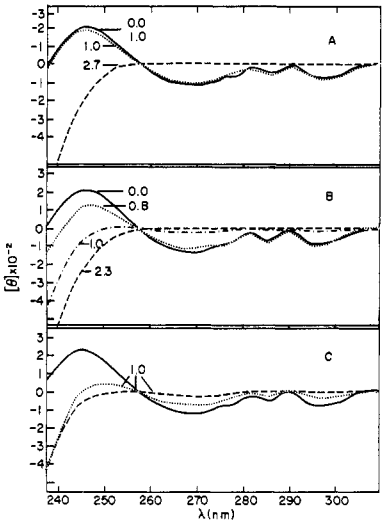

FIGURE 2: Near ultraviolet circular dichroism of carbonic anhydrase at pH 6, 25°. Numbers next to curves give the molar concentration of guanidine hydrochloride. Part A, holoenzyme; part B, apoenzyme. Dotted curve, part A, is for sample of enzyme originally denatured in 4.0 M guanidine hydrochloride, which was diluted to 1.0 M guanidine hydrochloride and allowed sufficient time to renature. Curves in part C illustrate effect of zinc on the apoenzyme; all curves, concentration of guanidine hydrochloride reduced by dilution from 4.0 M to 1.0 M; solid curve, 1 equiv of Zn^{2+} added before reduction of guanidine hydrochloride concentration; dashed curve, no Zn^{2+} added (sufficient time allowed for both samples to come to refolding equilibrium as judged by kinetic studies illustrated in Figure 4); dotted curve, 1 equiv of Zn^{2+} added to sample represented by dashed curve and measurements made after several months at 4°.

Transition from the Native to the Denatured State. Guanidine hydrochloride denaturation of carbonic anhydrase results in dramatic changes in circular dichroism (Figure 2). The characteristic Cotton effects in the aromatic region of the spectrum are lost. Below 250 nm the circular dichroism of the denatured enzyme is negative. The denaturation of the zinc-free apoenzyme, judged by changes in circular dichroism, follows a course similar to the zinc enzyme; however, it is interesting to note that for the apoenzyme the transition is complete slightly above 1.0 M guanidine hydrochloride whereas for the

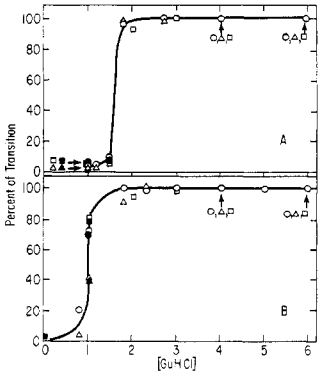

FIGURE 3: Guanidine hydrochloride (Gdn·HCl) denaturation transition at pH 6, 25°. Curve A, carbonic anhydrase; curve B, apocarbonic anhydrase. Protein concentration usually *ca.* 3×10^{-5} M. Open symbols represent denaturation; filled symbols represent renaturation after exposure to 4 M GuHCl. The transitions were followed by measuring the change in $[\theta]_{247}$, (○) or (●); ϵ_{292} (△) or (▲); and *p*-nitrophenyl acetate activity (see text), (□) or (■).

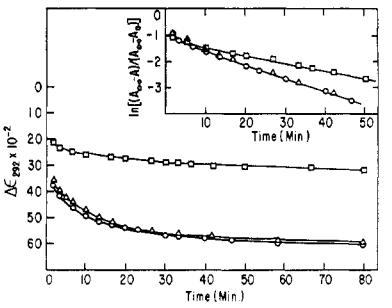

FIGURE 4: Rate of renaturation, as judged by changes in $\Delta\epsilon_{292}$, at pH 6, 25° following dilution of guanidine hydrochloride from 4.0 M to 1.0 M: (○) zinc enzyme; (△) apoenzyme to which 1 equiv of Zn^{2+} added before dilution of guanidine hydrochloride; (□) apoenzyme. Reference cell contained denatured enzyme in 4.0 M guanidine hydrochloride so that at time zero, $\Delta\epsilon_{292} = 0$. Protein concentration in the kinetic experiments ranged from *ca.* 3×10^{-6} M to 3×10^{-5} M. The insert gives first-order plots of the absorbance data where A_0, A, and A_∞ are absorbances at $t = 0$, t, and ∞ respectively.

enzyme denaturation has not yet begun at this concentration of denaturant, and is complete only above 1.8 M guanidine hydrochloride. However, on completion of the denaturation transition, the circular dichroism of the holoenzyme is identical with that of the apoenzyme. At the lower concentrations of guanidine hydrochloride the denaturation is relatively slow; consequently care was taken to allow enough time for equilibrium to be reached. The circular dichroism was usually measured after 30 min and then checked again after several weeks storage at 5°.

The denaturation of carbonic anhydrase was also followed by measuring the decrease in esterase activity, using *p*-nitrophenyl acetate as substrate. Because the guanidine hydrochloride itself was found to inhibit the *p*-nitrophenyl acetate activity (at concentrations well below those required to denature the enzyme) its concentration was reduced by a 200-fold dilution with water before assaying. The changes in esterase activity determined by this method parallel the changes in circular dichroism. Figure 3 compares the denaturation transition determined by the two methods. It should be pointed out this method of determining decreases in esterase activity is a complex one because some refolding occurs rapidly on dilution; however, a major phase of the refolding reaction is slow compared to the few minutes necessary to carry out the assay (Figure 4).

Denaturation curves for the apo- and holoenzyme determined from changes in circular dichroism, ultraviolet difference spectra, and esterase activity (Figure 3) appear to be independent, albeit within a fairly wide uncertainty range, of the method used to follow the denaturation transition.

Recovery of the Native Conformation. On dilution of the guanidine hydrochloride from 4.0 M to 1.0 M the denatured enzyme refolds. If an equivalent of Zn(II) is present during the refolding process, an enzyme evidently native as judged by its circular dichroism and ultraviolet absorption spectrum is regenerated. There is essentially no difference between the ultraviolet spectrum of refolded and native enzyme (Figure 1), indicating that the aromatic side chains have returned to environments similar or identical with those in the native structure. In particular, the disappearance of the 292-nm difference peak on refolding suggests that all six tryptophan side chains which were exposed on denaturation have returned to the interior of the protein. The circular dichroism of the re-

folded enzyme in the region from 240 to 320 nm (Figure 2) indicates that the interactions of the aromatic side chains with dissymetrical regions of the enzyme are the same in native enzyme and enzyme refolded in the presence of an equivalent of Zn(II).

Reversibility of the denaturation–renaturation reaction is further illustrated by the transition curves shown in Figure 3. For a given concentration of denaturant the spectral properties are the same regardless of whether denatured enzyme is refolded or native enzyme is unfolded, provided of course enough time is allowed for equilibrium to be reached.

The esterase activity (*p*-nitrophenyl acetate as substrate) of carbonic anhydrase solutions prepared by a 1:200 dilution with water of denatured enzyme in 4 M guanidine hydrochloride was determined. In all cases refolding, under these conditions, resulted in recovery of greater than 95% of the original enzymatic activity.

The rate of refolding is relatively slow and refolding follows a complex kinetic pathway. For example, Figure 4 shows the kinetics of refolding in a solution of 1.0 M guanidine hydrochloride. The reaction was followed by monitoring changes in absorbance at 292 nm, although the same results are obtained at other wavelengths. On refolding, the absorbance at 292 nm initially increases very rapidly; this initial burst, which is essentially complete in a few seconds, is followed by a process which gives a curved semilog plot. The initial burst accounts for about one-half of the total change in absorbance. The remaining slower phases of the refolding process are half completed in about 10 minutes.

In the renaturation experiments the kinetic and equilibrium results were the same for the native enzyme, apoenzyme reactivated with Zn^{2+} before denaturation, and denatured apoenzyme to which an equivalent of Zn^{2+} was added before renaturation.

In the absence of Zn(II), the apoenzyme also refolds if the concentration of guanidine hydrochloride is reduced by dilution. In 1.0 M guanidine hydrochloride, refolding follows a complex kinetic pathway similar to that observed in the presence of Zn(II), although both the rate and extent of reaction are lower (Figure 4). Experiments at other guanidine hydrochloride concentrations show that although the absence of zinc has resulted in a shift of the denaturation–renaturation transition to lower guanidine hydrochloride concentration, the transition is still reversible (Figure 3). Furthermore, on completion of the renaturation transition, fully active enzyme is rapidly regenerated by the addition of 1 equiv of Zn^{2+}, as judged by the experiments in which a solution of the denatured apoenzyme in 4 M guanidine hydrochloride was diluted 1:200 with water; the apoenzyme allowed to refold; 1 equiv of Zn^{2+} added; and the solution immediately assayed with *p*-nitrophenyl acetate as substrate.

These experiments indicate that the presence of zinc is not required for refolding to the native structure, and that fully active enzyme can be regenerated by the addition of 1 equiv of Zn^{2+}, either before or after the refolding transition. However under some conditions the absence of Zn(II) during the refolding process has important kinetic consequences. This is illustrated by experiments in which Zn^{2+} was added to a solution of the partially denatured apoenzyme in 1.0 M guanidine hydrochloride. The results of these experiments were the same for solutions obtained either by denaturing enzyme originally in water or renaturing enzyme originally in 4.0 M guanidine hydrochloride. Although the addition of Zn^{2+} at this stage appears to lead to recovery of the native structure and activity, the transition now occurs at an extremely low rate, with the result that at 4° there is no detectable change in circular dichroism after 12 hr and only a small change after 2 weeks. Even after several months the transition is only partially completed (Figure 2). Dilution and immediate assay with *p*-nitrophenyl acetate as substrate indicate that at this stage about 30% of the enzymatic activity has been recovered.

Discussion

The results of this study indicate that like many other enzymes, bovine carbonic anhydrase B is denatured by guanidine hydrochloride and that it refolds spontaneously upon removal of the denaturing condition. Judged by circular dichroism and ultraviolet difference spectroscopy, guanidine hydrochloride denaturation carries both the native and the apoenzyme from the same initial to the same final state, exposing six buried tryptophan side chains and disrupting interactions of the aromatic side chains with dissymmetrical regions of the enzyme. Presumably on completion of the denaturation transition both native and apoenzyme contain no noncovalent structure (*cf.* review by Tanford, 1968), with the result that the specific zinc binding site of the enzyme is destroyed. Tanford (1968) points out that all proteins which have been studied in high concentrations of guanidine hydrochloride have been found to contain no residual noncovalent structure. In this case, since carbonic anhydrase contains no disulfide bonds (Nyman and Lindskog, 1964), loss of all noncovalent structure would result in a linear random coil, in which event denatured apoenzyme to which an equivalent to Zn^{2+} had been added would be expected to behave exactly the same as denatured native enzyme. This is in accord with all our experience.

The kinetic observations indicate that refolding, both in the presence and absence of Zn(II), proceeds through intermediates which contribute to the observed ultraviolet absorbance. A two state transition involving only the denatured and native state would give linear semilog plots which extrapolate through zero rather than those observed in Figure 4. Additional evidence for the formation of intermediate conformational states during the transition has been reported by Wong and Tanford (1970).

Refolding leads to the recovery of the original conformation, as judged by ultraviolet difference spectroscopy and circular dichroism. The presence of zinc is not required, demonstrating that the preferred conformation is the same with or without the metal. The observed reversibility of the denaturation–renaturation transitions provides strong evidence that this preferred conformation represents a thermodynamic state of minimum free energy for both the apo- and holoenzyme, not simply a local minimum, despite the fact that measurements of Lindskog and Malmström (1962) lead to $\Delta G' = -16.4$ kcal mole^{-1} (standard state for hydrogen ions at activity of 10^{-7} M) for the binding of Zn^{2+} to the apoenzyme in dilute salt solution at 25° and pH 7.

As mentioned earlier, renaturation from the randomly coiled conformation occurs readily in 1.0 M guanidine hydrochloride if Zn(II) is present during the initial stages of the refolding process, whereas it occurs at an extremely low rate if Zn(II) is added later. While the present study does not provide any detailed information on this effect of zinc, the results are consistent with the view that, at least in 1.0 M guanidine hydrochloride, intermediate(s) are formed in the absence of zinc which can not be readily converted to the native conformation, suggesting that Zn(II) is bound during the initial

steps of the folding of the polypeptide chain and thus influences the pathway of the reaction although it does not affect the final conformational state. Although admittedly 1.0 M guanidine hydrochloride is far from the environment a nascent protein molecule experiences as it separates from the ribosome, the observations reported here point up the possibility that with other proteins containing metals or noncovalently bound prosthetic groups, a similar situation may exist under more physiological conditions.

References

Beychok, S., Armstrong, J. McD., Lindbrow, C., and Edsall, J. T. (1966), *J. Biol. Chem. 241*, 5150.

Brewer, J. M., Spencer, T. E., and Ashworth, R. B. (1968), *Biochim. Biophys. Acta 168*, 359.

Coleman, J. E. (1965), *Biochemistry 4*, 2644.

Coleman, J. E. (1968), *J. Biol. Chem. 243*, 4574.

Donovan, J. W. (1964), *Biochemistry 3*, 67.

Donovan, J. W. (1969), *in* Physical Principles and Techniques of Protein Chemistry, Leach, S. J., Ed., New York, N. Y., Academic Press, p 102.

Fridborg, K., Kannan, K. K., Liljas, A., Lundin, J., Strandberg, B., Strandberg, R., Tilander, B., and Wirén, G. (1967), *J. Mol. Biol. 25*, 505.

Hamaguchi, K., Kurono, A., and Goto, S. (1963), *J. Biochem. (Tokyo) 54*, 259.

Henkens, R. W., and Lochmüller, C. (1970), Abstracts, Combined 22nd Southeastern 26th Southwestern Regional Meeting of the American Chemical Society, New Orleans, La.

Henkens, R. W., and Sturtevant, J. M. (1968), *J. Amer. Chem. Soc. 90*, 2669.

Henkens, R. W., Watt, G. D., and Sturtevant, J. M. (1969), *Biochemistry 8*, 1874.

Kernohan, J. C. (1965), *Biochim. Biophys. Acta 96*, 304.

Lindskog, S. (1960), *Biochim. Biophys. Acta 39*, 218.

Lindskog, S. (1963), *J. Biol. Chem. 238*, 945.

Lindskog, S., and Malmström, B. G. (1962), *J. Biol. Chem. 237*, 1129.

Lindskog, S., and Nyman, P. O. (1964), *Biochim. Biophys. Acta 85*, 462.

Nyman, P. O., and Lindskog, S. (1964), *Biochim. Biophys. Acta 85*, 141.

Reynolds, J. A., and Schlesinger, M. J. (1969), *Biochemistry 8*, 588.

Tanford, C. (1968), *Advan. Protein Chem. 23*, 121.

Teipel, J. W., and Koshland, D. E., Jr. (1971), *Biochemistry 10*, 792.

Timasheff, S. N. (1970), *Enzymes 2*, 371.

Wong, K. P., and Tanford, C. (1970), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 29*, 335.