Clin. Med. 52, 312.
Jensen, M., Oski, F. A., Nathan, D. G., and Bunn, H. F. (1975), *J. Clin. Invest. 55,* 469.
Jones, R. T. (1970), *Methods Biochem. Anal. 18,* 205.
Jornwall, H. (1975), *J. Theor. Biol. 55,* 1.
Kilmartin, J. V., and Rossi-Bernardi, L. (1969), *Nature (London) 22,* 1243.
Lorkin, P. A., Stephens, A. D., Beard, M. E., Wrigley, P. F. M., Adams, L., and Lehman, H. (1975), *Br. Med. J. 4,* 200.
Malik, N., and Berrie, A. (1972), *Anal. Biochem. 49,* 173.
Marchis-Mouren, G., and Lipmann, F. (1965), *Proc. Natl. Acad. Sci. U.S.A. 53,* 1147.
Moo-Penn, W. F., Jue, D. L., Bechtel, K. C., Johnson, M. H., Schmidt, R. M., McCurdy, P. R., Fox, J., Bonaventura, J., Sullivan, B., and Bonaventura, C. (1976), *J. Biol. Chem. 251,* 7557.
Nigen, A. M., and Manning, J. M. (1975), *J. Biol. Chem. 250,* 8248.
Perutz, M. F., Muirhead, H., Mazzarella, L., Crowther, R. A., Greer, J., and Kilmartin, J. V. (1969), *Nature (London) 222,* 1240.
Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10,* 467.
Reynolds, C. A., and Huisman, T. H. J. (1966); *Biochim. Biophys. Acta 130,* 541.
Riggs, A. F., and Wolbach, R. A. (1956), *J. Gen. Physiol. 39,* 585.
Schmidt, R. M., and Brosious, E. M. (1976), Basic Laboratory Methods of Hemoglobinopathy Detection, 8th ed, DHEW Publication No. CDC 76-8266, Atlanta, Ga., DHEW-PHS.
Schneider, R. G. (1974), *Clin. Chem. (Winston-Salem, N.C.) 20,* 1111.
Schroeder, W. A. (1972), *Methods Enzymol. 25,* 203.
Schroeder, W. A., Gua, J. T., Matsuda, G., and Fenninger, W. D. (1962), *Biochim. Biophys. Acta 63,* 532.
Schulten, H. R., and Beckey, H. D. (1972), *Org. Mass Spectrom. 6,* 885.
Shamsuddin, M., Mason, R. G., Ritchey, J. M., Honig, G. R., and Klotz, I. M. (1974), *Proc. Natl. Acad. Sci. U.S.A. 71,* 469.
Smyth, D. C. (1967), *Methods Enzymol. 11,* 216.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Wüthrich, K. (1976), in NMR in Biological Research: Peptides and Proteins, New York, N.Y., Elsevier North-Holland.

Effect of Pressure upon the Fluorescence of Various Flavodoxins†

A. J. W. G. Visser,‡ T. M. Li,§ H. G. Drickamer, and Gregorio Weber*

ABSTRACT: The effects of hydrostatic pressure in the range of 10^{-3} to 11 kbar on the fluorescence of flavodoxins from *Peptostreptococcus elsdenii, Desulfovibrio vulgaris, Azotobacter vinelandii,* and *Clostridium* MP were investigated. The first three flavoproteins showed under high pressure enhancements of flavin fluorescence of over 50 times resulting from the release of flavin mononucleotide from the protein complex. The Clostridial flavodoxin showed a very much smaller fluorescence change. At pH 7.5 the high-pressure fluorescence changes of the flavodoxins of *D. vulgaris* and *P. elsdenii* were not reversed by decompression, but in *A. vinelandii* the pressure changes were over 80% reversible. At pH 5 over 80% reversibility was restored to the flavodoxins of *D. vulgaris* and *P. elsdenii*, although the pressure dependence of the fluorescence changes was very similar in the reversible and irreversible cases. The midpoint pressures in the reversible reactions were 4.7 kbar (*D. vulgaris*), 8.7 kbar (*P. elsdenii*), and 10.6 kbar (*A. vinelandii*) indicating specific differences in the flavin binding regions. Apparent volume changes in these reactions were 65–75 mL/mol indicating participation of a large fraction of the protein in the pressure-induced changes. The irreversible changes are not related to protein aggregation and are believed to result from a pressure-dependent covalent modification, not yet characterized, of the flavin binding region of the protein.

Fluorescence techniques can be conveniently applied to study denaturation processes in proteins by means of hydrostatic pressure (Li et al., 1976a,b). Following the investigation on the effect of pressure upon the flavin-binding protein (Li et al., 1976a) we have investigated the effects of high pressure upon another class of flavoproteins, the flavodoxins. Flavodoxins are single peptide-chain proteins of relatively low molecular weight (15 000–23 000) which function as low-potential electron carriers in a variety of organisms (Knight et al., 1966; Shethna et al., 1966; Mayhew and Massey, 1969; Dubourdieu and LeGall, 1970; Mayhew, 1971a). The flavodoxins contain a single, noncovalently attached molecule of flavin mononucleotide (FMN) per molecule of protein. From the large flavodoxin family, four of them isolated from different bacterial strains were selected for this study, namely flavodoxin from *Peptostreptococcus elsdenii, Clostridium* MP, *Desulfovibrio vulgaris,* and *Azotobacter vinelandii.* These flavodoxins are well characterized. The amino acid sequences of the first three are known (Tanaka et al., 1973, 1974; Dubourdieu et al.,

† From the Department of Biochemistry (G.W.) and Department of Chemical Engineering (H.G.D.), School of Chemical Sciences and Materials Research Laboratory, University of Illinois, Urbana, Illinois 61801. *Received May 6, 1977.* Supported by Grant No. GM 11223, U.S. Public Health Service, to G. W. and by the Energy Research and Development Administration under Contract No. E(11-1)-1198 to H.G.D. A.G.W.G.V. was a recipient of a NATO Science Fellowship supplied by the Netherlands Organization for the Advancement of Pure Research (Z.W.O.).

‡ Present address: Department of Biochemistry, Agricultural University, Wageningen, The Netherlands.
§ Present address: Institute of Cancer Research, Fox Chase, Philadelphia, Pa. 19111.

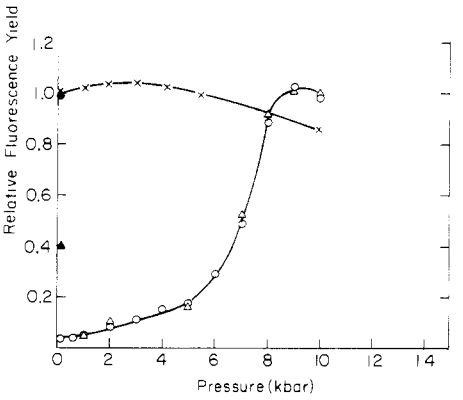

FIGURE 1: Relative fluorescence yield vs. pressure of FMN and 8.0 μM *P. elsdenii* flavodoxin at 23 °C in 0.1 M Tris-HCl buffer pH 7.5: (×) FMN alone; (○) FMN in flavodoxin; (△) protein fluorescence. Solid symbols refer to data obtained after compression to 10 kbar and decompression. Flavin and protein were excited at 375 and 295 nm, respectively.

1973). The crystal structures of the *Clostridium* MP and *D. vulgaris* flavodoxins have been reported at 1.9- and 2-Å resolution (Burnett et al., 1974; Watenpaugh et al., 1973). The binding sites differ markedly in the two cases while the overall structure is quite similar. Except for the *A. vinelandii* and *D. vulgaris* flavodoxins, the phosphate group of FMN is crucial for binding. The *A. vinelandii* flavodoxin is somewhat larger (mol wt 23 000) than the other three (15 000–17 000), but the amino acid compositions are quite similar (Edmondson and Tollin, 1971). In the four flavodoxins both protein fluorescence and FMN fluorescence are largely or almost completely quenched. The situation is in this respect quite similar to that of the flavin complexes of the riboflavin-binding protein (Li et al., 1976a) and we may expect the study of pressure effects upon the fluorescence of the flavodoxins to provide information analogous to that case.

Materials and Methods

Flavodoxin from *P. elsdenii* was a gift of Dr. V. Massey, University of Michigan, Ann Arbor, Mich. Flavodoxins from *Clostridium* MP, *D. vulgaris*, and *A. vinelandii* were kindly provided by Dr. S. G. Mayhew, Agricultural University, Wageningen, The Netherlands. Prior to use, the protein solutions, containing approximately 0.5 mg/mL, were dialyzed for about 48 h against several changes of the appropriate buffer. The solution was then further diluted with the same buffer to give an absorbancy of 0.1 at 450 nm, which corresponds to 10 μM protein concentration. Published values of the absorption coefficient were used to determine the exact concentration of protein in each case (Hinkson and Bulen, 1967; Mayhew and Massey, 1969; Dubourdieu and LeGall, 1970; Mayhew, 1971a). The apoflavodoxin of *P. elsdenii* was prepared according to Mayhew (1971b). Removal of free flavin and KBr was obtained by dialysis against 0.1 M (pH 7.0) sodium phosphate buffer containing 3×10^{-4} M EDTA. This was followed by dialysis against the appropriate buffer. The concentration was determined using the absorption coefficient of 26 700 M^{-1} cm^{-1} at 270 nm (Mayhew, 1971b). FMN was purified from commercial FMN (Sigma) according to the procedure of Massey and Swoboda (1963). The buffers used in the experiments were 0.1 M Tris-HCl (pH 7.5), prepared from Trizma base (Sigma), and 0.1 M sodium acetate–acetic acid (pH 5.0). EDTA (3×10^{-4} M) was added to both solutions to prevent metal-catalyzed oxidation of sulfhydryl groups. The pH value of Tris-HCl buffer is almost pressure invariant, whereas the pH of acetate buffers is expected to decrease by less than one unit at a pressure of 6.5 kbar (Neuman et al., 1973). Where not mentioned, all chemicals were of reagent grade and used without further purification. Sedimentation velocity experiments were carried out with a Beckman Model E analytical ultracentrifuge equipped with a UV scanning system. Optical densities were obtained with a Zeiss PMQ-II spectrophotometer and absorption spectra with a Beckman Acta MVI recording spectrophotometer.

Fluorescence spectra at atmospheric pressure were recorded on a scanning photon-counting spectrofluorimeter described by Jameson et al. (1976). Fluorescence yields and spectra under pressure were obtained as already described (Li et al., 1976a,b, and literature cited therein). For observation of flavin and protein fluorescence, excitations at 375 and 295 nm, respectively, were employed.

Results

P. elsdenii Flavodoxin at pH 7.5. Figure 1 shows a plot of relative intensity of flavin fluorescence (maximum at 525 nm) against pressure. The fluorescence intensity increases steadily between atmospheric pressure and 5 kbar and sharply in the region of 6–9 kbar. At the highest pressures the flavin is dissociated resulting in a 50-fold enhancement of fluorescence. However, these changes are not reversible; the fluorescence intensity remains at the high level and the characteristic structure of the visible absorption spectrum of flavodoxin is lost after decompression. Irreversible changes in fluorescence were also observed when, in a separate experiment, the pressure was increased to 4.5 kbar, at which point the fluorescence had risen to only one-fifth of the maximum value, and then released. An experiment in which dithiothreitol had been added as a potential sulfhydryl protector gave results similar to those shown in Figure 1. In none of these experiments was a precipitate of protein, or other signs of aggregation, seen after decompression and opening of the high-pressure cell. Several experiments carried out with the compressed protein lead to the following conclusions. (1) The pressurized protein, in contrast to the native flavodoxin, lost the flavin readily upon dialysis. (2) The sedimentation onstants of native and pressurized flavodoxins were identical ($s_{20,w}$ = 1.96 and 1.92 S, respectively, at a protein concentration of 0.37 mg/mL). Thus, no aggregation took place under pressure. (3) The ability of the protein to bind FMN could not be restored by treating the pressurized protein either with 6 M urea containing 10^{-4} M mercaptoethanol or with 5% trichloroacetic acid, followed by dialysis or dilution into neutral phosphate buffer. The native flavodoxin is known to undergo fully reversible denaturation under these conditions (Mayhew and Massey, 1969). The tryptophan fluorescence of the holoprotein was also followed as a function of pressure and the results are included in Figure 1. By use of a relative scale for these changes it is observed that the protein fluorescence increases in much the same way as the flavin fluorescence. The protein emission maximum shifts from 331 to 341 nm. Both this spectral shift and the changes in intensity are irreversible indicating that the prosthetic group is indeed dissociated from the protein and not bound in a highly fluorescent form.

The results obtained with the apoflavodoxin are displayed in Figures 2 and 3. The intensity decreases smoothly up to 10 kbar and the change in intensity is not reversed upon release of pressure. The position of the fluorescence maximum of apoflavodoxin at pH 4.9 is unaffected by pressure (Figure 3). After decompression the apoprotein totally loses its ability to bind FMN.

Other Flavodoxins at pH 7.5. The results of these experiments are summarized by Figure 4. The striking observation

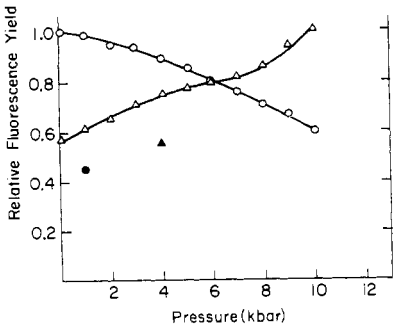

FIGURE 2: Relative protein fluorescence yield vs. pressure of 8.3 μM *P. elsdenii* apoflavodoxin at 23 °C: (○) in 0.1 M Tris-HCl buffer at pH 7.5; (△) in 0.1 M sodium acetate buffer at pH 4.9. Solid symbols were data obtained when pressure was decreased after reaching a pressure of 10 kbar. Excitation wavelength was at 295 nm.

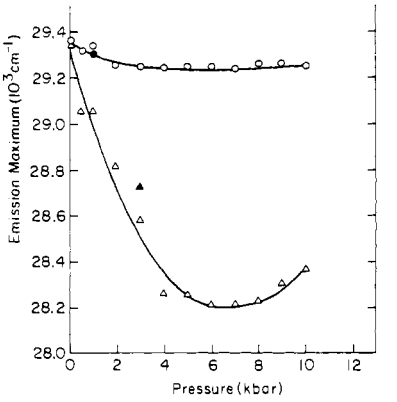

FIGURE 3: Emission band shifts vs. pressure of *P. elsdenii* apoflavodoxin. Conditions and symbols are as in Figure 2.

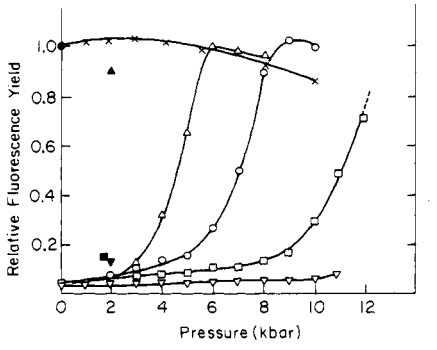

FIGURE 4: Relative fluorescence yields vs. pressure of FMN in several flavodoxins at 23 °C in 0.1 M Tris-HCl buffer at pH 7.5: (△) 9.0 μM *D. vulgaris* flavodoxin; (○) 8.0 μM *P. elsdenii* flavodoxin; (□) 8.8 μM *Clostridium* MP flavodoxin; (▽) 11.5 μM *A. vinelandii* flavodoxin; (×) FMN alone. Solid symbols and ▣ refer to data obtained after decompression after reaching either maximum intensity or 11 kbar. Excitation wavelength was at 375 nm.

is that the midpoint pressure for the sharp fluorescence rise, $p_{1/2}$, is different for all four flavodoxins. The relative intensity of the fluorescence from the *D. vulgaris* protein increases slowly in the range below 3 kbar, rapidly in the range 3–6 kbar. In the *A. vinelandii* protein the slow rise continues up to 9 kbar and $p_{1/2}$ is estimated at 10.5 kbar. In the *Clostridium* MP flavodoxin the effect of pressure is very limited. The intensity of fluorescence starts to rise only at high pressure (11 kbar). The changes in fluorescence of these proteins are not reversible, except for the flavodoxin of *A. vinelandii* which shows 83% recombination with FMN after reducing the pressure. The

TABLE I: Transition Midpoint Pressure, $p_{1/2}$, and Reversibility upon Pressure-Induced Denaturation of Flavodoxins at Two Different pH Values.

Flavodoxin from	pH 7.5		pH 5.0	
	$p_{1/2}$ (kbar)	Reversibility	$p_{1/2}$ (kbar)	Reversibility
D. vulgaris	4.5	−	4.8	+
P. elsdenii	7.1	−	8.6	+
Clostridium MP	>11.0	−	>11.0	+
A. vinelandii	10.5	+		

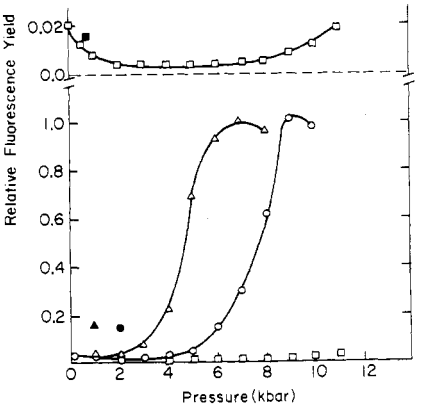

FIGURE 5: Relative fluorescence yield vs. pressure of FMN in several flavodoxins at 23 °C in 0.1 M sodium acetate buffer at pH 5.0: (△) 10.7 μM *D. vulgaris* flavodoxins; (○) 8.2 μM *P. elsdenii* flavodoxin; (□) 12.4 μM *Clostridium* MP flavodoxin. Symbols and excitation wavelength are as in Figure 4.

characteristic flavoprotein spectrum was practically unaltered in the *Clostridium* MP and *A. vinelandii* proteins. $p_{1/2}$ values and reversibility characteristics are collected in Table I.

Experiments at pH 5.0. In contrast to the experiments at pH 7.5, the results obtained at pH 5.0 indicate largely reversible pressure-induced denaturation. This is illustrated in Figure 5 which collects all the observations at this pH value. Based on comparison with free flavin fluorescence we estimate that 82–85% of the FMN recombines with *P. elsdenii* and *D. vulgaris* proteins after decompression. The relative fluorescence yield of the *P. elsdenii* flavodoxin is almost constant over a large pressure range and its $p_{1/2}$ value is shifted to higher pressure (8.6 kbar) from the value at pH 7.5. The $p_{1/2}$ value for *D. vulgaris* is also slightly higher (4.8 kbar). Even the small changes observed in the weak fluorescence of flavodoxin from *Clostridium* MP in the range below 11 kbar show improved reversibility, and the prosthetic group of this protein is not dissociated. The structured visible absorption band of the flavodoxins is virtually restored on decompression.

The effect of pressure on the fluorescence of the apoflavodoxin of *P. elsdenii* at pH 5.0 is the opposite of that observed at pH 7.5, as is made clear in Figures 2 and 3. The ultraviolet (UV) fluorescence yield increases gradually with pressure up to 7 kbar and then more rapidly between 7 and 10 kbar (Figure 2). The latter change is in agreement with the sharp increase in fluorescence intensity observed in the holoproteins. Upon increasing the pressure the emission maximum is shifted to 353 nm (28 330 cm^{-1}) above 5 kbar (Figure 3). This red shift indicates exposure of the tryptophans to the solvent. Both shift and intensity changes are fully reversible. The decompressed protein was able to recombine with FMN although some binding capacity was lost as compared with the untreated apoprotein.

TABLE II: Volume Change upon Denaturation, ΔV_{den}, and Free-Energy Change for Denaturation, $p_{1/2}\Delta V_{den}$, for Flavodoxins.

	ΔV_{den} (mL/mol)	$p_{1/2}\Delta V_{den}$ (kcal/mol)
D. vulgaris	−63	7.5
P. elsdenii	−74	15.7
A. vinelandii	−64	16.4

Both the establishment of equilibrium after the increase in pressure in the region of sharp transitions and the return to a stationary condition after release of the pressure were slow processes, consuming between 15 and 60 min in the different cases.

Discussion and Conclusions

Inspection of the curves in Figures 4 and 5 shows in three out of the four flavodoxins two regions of increase in flavin fluorescence with pressure. In the first region changes are of the order of 1%/kbar. In the high-pressure region (4–11 kbar) FMN fluorescence changes by 30%/kbar. The origins of the changes in the lower pressure region are not easy to interpret as they may involve a shift in the binding equilibrium of the flavin by the protein, as well as small changes in protein conformation at the binding site leading to decreased quenching of the fluorescence of bound flavin. On the other hand, the much larger change is easily interpreted as resulting from the release of FMN due to a large change in protein conformation. Here we can exclude a simple shift in the equilibrium of flavin and protein by inspection of Table II giving the apparent volume changes in the reaction that gives rise to flavin release. Using the coarse, but nonetheless reliable rule that a change, ΔV, in noncovalent interactions arises from an involved volume of the order of $100\Delta V$ or more (Li et al., 1976b) we conclude that the change in protein conformation that results in flavin release must involve a protein region of 5000 to 10 000 daltons. It is interesting to notice that the midpoint pressures of the regions of rapid change in flavin fluorescence are not too different at the pHs of 7.5 and 5.0 although at the former value there is in general irreversibility of the changes, and at the latter there is over 80% reversibility. From this we conclude that the reaction responsible for the irreversible nature of the changes is quite independent of the pressure-induced change in conformation that leads to flavin release. The experiments described in this paper lead to two important conclusions. First, the pressure effects can reveal unmistakable specific differences among a family of enzymes which have a great deal in common. These differences have their counterpart in the structural arrangement revealed in two of them by the x-ray crystallographic studies. In *D. vulgaris* protein the flavin is flanked by tyrosine and tryptophan, while in *Clostridium* MP flavodoxin a methionine and a tryptophan residue are at both sides of the isoalloxazine ring system (Burnett et al., 1974; Watenpaugh et al., 1973). Secondly, it is possible to demonstrate irreversibility of the pressure effects at one pH value and overall reversibility at another, the former not resulting from trivial protein aggregation. Although it has often been suggested that a protein in solution could exist in different metastable conformations no clear experimental evidence is known to the authors that would favor that hypothesis. For this reason we believe that the observed irreversibility arises from covalent chemistry, and a detailed examination of the pressured preparations will be carried out in the future to test this possibility.

Acknowledgments

We thank Drs. V. Massey and S. G. Mayhew for generous gifts of flavodoxins.

References

Burnett, R. M., Darling, G. D., Kendall, D. S., DeQuesne, M. E., Mayhew, S. G., Smith, W. W., and Ludwig, M. L. (1974), *J. Biol. Chem. 249,* 4383.

Dubourdieu, M., and LeGall, J. (1970), *Biochem. Biophys. Res. Commun. 30,* 965.

Dubourdieu, M., LeGall, J., and Fox, J. L. (1973), *Biochem. Biophys. Res. Commun. 52,* 1418.

Edmondson, D. E., and Tollin, G. (1971), *Biochemistry 10,* 124.

Hinkson, J. E., and Bulen, W. A. (1967), *J. Biol. Chem. 242,* 3345.

James, T. L., Ludwig, M. L., and Cohn, M. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3392.

Jameson, D. M., Spencer, R. D., and Weber, G. (1976), *Rev. Sci. Instrum. 47,* 1034.

Knight, E., d'Eustachio, A. J., and Hardy, R. W. F. (1966), *Biochim. Biophys. Acta 113,* 626.

Li, T. M., Hook, J. W., Drickamer, H. G., and Weber, G. (1976a), *Biochemistry 15,* 3205.

Li, T. M., Hook, J. W., Drickamer, H. G., and Weber, G. (1976b), *Biochemistry 15,* 5571.

Massey, V., and Swoboda, B. E. P. (1963), *Biochem. Z. 338,* 474.

Mayhew, S. G. (1971a), *Biochim. Biophys. Acta 235,* 276.

Mayhew, S. G. (1971b), *Biochim. Biophys. Acta 236,* 289.

Mayhew, S. G., and Massey, V. (1969), *J. Biol. Chem. 244,* 794.

Neuman, R. C., Kaufmann, W., and Zipp, A. (1973), *J. Phys. Chem. 77,* 2687.

Shethna, Y. I., Wilson, P. W., and Beinert, M. (1966), *Biochim. Biophys. Acta 113,* 225.

Tanaka, M., Haniu, M., Yasunobu, N. T., and Mayhew, S. G. (1974), *J. Biol. Chem. 249,* 4393.

Tanaka, M., Haniu, M., Yasunobu, N. T., Mayhew, S. G., and Massey, V. (1973), *J. Biol. Chem. 248,* 4354.

Watenpaugh, N. D., Sieker, L. C., and Jensen, L. H. (1973), *Proc. Natl. Acad. Sci. U.S.A. 70,* 3057.