ACTA CHEMICA SCANDINAVICA 19 (1965) 1643–1651

Studies of Conformational Changes in Glyceraldehyde-3-Phosphate Dehydrogenase Accompanying its Catalytic Action

B. H. HAVSTEEN

*Department of Biochemistry, Cornell University, Ithaca, N.Y., USA**

The optical rotatory dispersion of glyceraldehyde-3-phosphate dehydrogenase apoenzyme, holoenzyme (with NAD),** and G3P-hemimercaptalenzyme were measured in the range: 300–700 mμ at pH 7.7–8.4 at 23°C in 0.15 M salt solution (0.001 M in EDTA). The dispersion parameters λ_c, K and b_0 were calculated and used to estimate the helical content. A tentative interpretation of the observed changes in dispersion parameters in terms of conformational and other changes was made:

a. The helical content (as represented by b_0) of the apoenzyme is pH-dependent in the range: pH 7.7–8.4, but the solvation (K) is not.

b. Even a low concentration (6×10^{-4} M) of the nonpolar compound α-thioglycerol affects both the helical content (b_0) and the solvation of the apoenzyme (K).

c. Stoichiometric binding of the cofactor NAD to the apoenzyme alters the helical content (b_0), but not the solvation. Addition of excess NAD causes no further changes except in the amplitude of the anomalous rotation (at 650 mμ).

d. Formation of the substrate G3P-enzyme complex from the apoenzyme changes both the helical content (b_0) and the solvation (K). Anomalous rotation was observed in two wavelength ranges: one at about 305 mμ, the other at about 650 mμ. The reference curve indicated that the former was due to a Zn-complex (probably a Cotton effect), while the latter might arise from an interaction involving the sulphur atom of cystein.

* Present address: Max Planck Institute of Physical Chemistry, Göttingen, Germany.

** *Abbreviations:* ATP: Adenosine-tri-phosphate; EDTA: Ethylene diamine tetraacetic acid; G3P: Glyceraldehyde-3-phosphate; G3PDH: Glyceraldehyde-3-phosphate dehydrogenase; m.r.: Molar ratio; NAD: Nicotin adenine dinucleotide; NADH: Reduced NAD; O.R.D.: Optical rotatory dispersion; 3-PGA: 3-Phospho-glycerate; PEP: Phospho enol pyruvate; Tris: Tris-(hydroxymethyl)-aminomethane; Apo-enz.: Protein moiety of the enzyme; Holo-enz.: Apoenzyme + cofactor; K_{stab}: Stability constant.

Considerable attention has in the last years been given to the possible role of conformational changes in enzymes in the course of their action. Evidence of the occurrence of such rearrangements is quite ample in some systems, especially in the catalysis of ester hydrolysis by α-chymotrypsin [1,2] and the action of *D*-amino acid oxidase.[3]

Though most evidence is available for the transferases, the view that the phenomenon is very general is gaining acceptance. This work was designed to enlarge the knowledge of the symmetry of a typical oxidation-reduction enzyme, its protein part *per se*, and its complexes with a cofactor (NAD) and a substrate (G3P). The latter is an intermediate in the catalytic mechanism.[4] Glyceraldehyde-3-phosphate dehydrogenase (G3PDH) was chosen because of its key position in glycolysis and because many of its properties already are quite well established.[5–11] Studies of G3PDH have a special interest because there are indications suggesting that this enzyme is subject to allosteric control, possibly through the binding of the second nicotinamide-adenine-dinucleotide molecule (NAD), which is not directly involved in the catalytic action. The binding could induce conformational changes in the active site independent of those which might be brought about by the substrate binding.[4,12]

Hvidt and Kägi [13] have found changes in the rate of deuterium-hydrogen exchange in yeast alcohol dehydrogenase on addition of NAD. This phenomenon, which was studied by infrared spectrophotometry, also indicates the occurrence of conformational changes in dehydrogenases following substrate binding.

Boyer [14] has already reported a change in the specific rotation of G3PDH after addition of NAD, but optical rotatory dispersion measurements are required to clarify whether the origin of the change in the optical rotation is a change in the amplitude of a Cotton effect arising from a helix, a solvation, a cofactor-apoenzyme complex, a metal ion-enzyme complex or a source which sofar has escaped attention. Elödi and Szabolcsi [15] made observations similar to those of Boyer.

EXPERIMENTAL

Materials. A suspension of crystals of yeast G3PDH in a solution containing NAD (molar ratio NAD/G3PDH = 5.9) and a low concentration of ammonium sulfate was donated by Dr. E. Racker, the Public Health Research Institute of the City of New York. The apoenzyme was isolated by chromatography at about 2°C on a short column (*ca.* 5 mm) of a mixture (1:1 by wt.) of active carbon and Sephadex G-25 dextran gel. The charcoal (Norit A, neutral) was purchased from Fisher Scientific Co. and was purified according to the method of Krimsky *et al.*[7] The dextran gel (Pharmacia, Uppsala, Sweden) was washed in water before use. The activity of the enzyme toward G3P was determined by the method of Krimsky *et al.*[7] The absorbancy ratio: A_{280}/A_{260} was used as evidence of the absence of significant quantities of denatured protein and of impurities of purine and pyrimidine derivatives. This requirement is satisfied when the ratio has a high value. The best preparations had an A_{280}/A_{260}-ratio of about 2. A specific extinction at 280 mμ of 0.908 for a 0.1 % solution of the protein was used.[16]

The holoenzyme was prepared by reconstitution from the apoenzyme and excess NAD (6–23 moles NAD/mole enzyme).[17] The molecular weight was assumed to be 140 000.[18] The G3P was purchased from Boehringer & Söhne, Mannheim, Germany (grade A, assay: 99 %) as barium salt of the diethylacetal. The acetal was hydrolyzed and the Ba-ions were exchanged with protons by treatment of a suspension of 25 mg of the substance and 1.9 ml moist Dowex-50 resin (H-form) in 5 ml water for 3 min with intermittent shaking in a boiling water bath.

The hemimercaptal-enzyme complex was formed by addition of about 4000 fold G3P to the apoenzyme at pH 7.7 and room temperature. This forces the equilibrium:

$$G3P + {}^{-}S-G3PDH \rightleftharpoons {}^{-}G3P-S-G3PDH \ (K_{stab} = 2.2 \times 10^{-5} \text{ l/mole})$$

practically quantitatively (99.9 %) toward the complex.

After the measurement of the optical rotatory dispersion about 6 moles of NAD per mole of enzyme were added to form the holoenzyme and expel the product (3-phosphoglyceric acid (3-PGA)). The latter, as well as NADH and excess NAD, was removed by Sephadex chromatography. NADH is easily removed since the stability constant for its complex with the apoenzyme is far smaller than that of NAD and the binding site is the same.[6,21] NADH is a potent inhibitor of the acyl-enzyme formation and must therefore be removed, *e.g.* by the reaction:[19]

$$H^+ + CH_3-CO-COO^- + NADH \overset{\text{lactic dehydrogenase}}{\rightleftharpoons} CH_3-CHOH-COO^- + NAD^+$$

The completeness of the removal of products and cofactors was tested by a specific assay for 3-PGA [20] and by measurement of the absorbancy ratio (A_{280}/A_{260}). The latter must have a value near 2. After the measurement of the optical rotatory dispersion of the holoenzyme the activity of the enzyme was determined as described above.

The substrates, cofactors, ATP, pyruvate kinase, phosphokinase, and lactic dehydrogenase, were purchased from Boehringer & Söhne. The water was distilled, deionized and outboiled. All other reagents were Mallinckrodt chemicals of AR-grade.

Instruments. The optical rotatory dispersion measurements were made with a Rudolph High Precision spectropolarimeter with photoelectric attachment and rocking analyzer as previously described.[22] The temperature was kept constant within ± 0.3°C with circulating water. The measurements were made in a 2 dm quartz polarimeter cell with water jacket (Optical Cell Co., Kensington, Md., U.S.A.). The lamp was a Hanovia xenon-mercury concentrated arc lamp from Englehart Industries (Newark, N. J., U.S.A.).

All spectrophotometric measurements were made with a Cary-14 recording spectrophotometer (Applied Physics Corp., Monrovia, Calif.). The protein concentration in the polarimeter tube was 0.6–1.8 mg/ml. The pH-measurements were made with a Radiometer pH-meter model TTT1.

RESULTS

The dispersion curves were obtained by subtraction of the appropriate solvent curves from the directly observed data. The adherence of the data to the Drude law:

$$[\alpha] = K/(\lambda^2-\lambda_c^2)$$

where $[\alpha]$ = specific rotation at the wavelength (mμ), K = constant, λ = wavelength of incident light and λ_c = critical wavelength, was tested graphically in the range 300–700 mμ (Figs. 1–3). The data are summarized in Tables 1–4. Table 1 shows the experimental conditions, some criteria of the quality of the preparations and the characteristics of the anomalous rotation effects tentatively ascribed to Cotton effects.[28,29] The O.R.D. parameters and estimates if the helical content are presented in Table 2. The parameter b_0 was determined with the aid of the Moffitt equation:[23,24]

$$[m'] = [a_0\lambda_0^2/(\lambda^2-\lambda_0^2)] + [b_0\lambda_0^4/(\lambda^2-\lambda_0^2)^2]$$

where $[m']$ = mean residue rotation = $[\alpha]$ $(M_0/100)$ $(3/(n^2+2))$, $[\alpha]$ = specific rotation, M_0 = mean residue weight = mol.wt./total number of AA, AA = amino acid and n = refractive index.

The value of λ was chosen to be 212 mμ,[22] because this value gives a good fit of the data to the equation for poly-γ-benzyl-L-glutamate and for many

Table 1.

Protein	[Enz.] mg/ml	[NAD]/ [Enz.]^a	pH	Solvent	Temp. °C	Anomal. rot.^{d} λ of A_{max} mμ ∓ 1.	∓ 2.	$\frac{A_{280}}{A_{260}}$	Activity min^{-1} ^{b}
0	0	—	7.42	6×10^{-5} M $ZnCl_2$, 3×10^{-5} M NAD,	20.1 ± 0.2	310 ± 5	—	—	—
0	0	—	7.5	0.11 M KCl, 0.04 M tris	20.1 ± 0.2	310 ± 5	—	—	—
Apoenzyme	0.775	0	8.4	0.15 M KCl, 0.001 M EDTA	23.5 ± 0.5	310 ± 10	675 ± 30	1.66	1.8 × 10^6 F ^{e}
»	0.588	0	7.7	» »	23.5 ± 0.6	310 ± 10	675 ± 30	1.85	1.17 × 10^6 »
»	1.87	0	7.70	0.15 M KCl, 0.001 M EDTA, 6×10^{-4} M α-thio-glycerol	23.5 ± 0.4	310 ± 10	650 ± 30	1.50	2.50 × 10^6 »
Holoenzyme (w. NAD)	1.64	9.2	7.70	» 6×10^{-4} »	23.4 ± 0.5	312 ± 8	630 ± 30	—	1.43 × 10^6 »
» » »	1.06	22.5	7.70	» 6×10^{-4} »	23.2 ± 0.2	312 ± 8	600 ± 40	—	0.37 × 10^6 »
Hemimercaptal-enzyme ^{c}	1.70	0	7.7	0.15 M KCl, 0.001 M EDTA, 0.081 M G3P	23.4 ± 0.1	312 ± 8	640 ± 30	1.29	0.012 × 10^6 »

a. Molar ratio based on a molecular weight of 140 000 of the protein.

b. Racker found: 8.0×10^6 min^{-1} at 27°C, pH 8.6, assuming a mol. wt. of 130 000.

c. $[E]/[ES] = K_S/[S] \simeq K_M/[S] = 2.2 \times 10^{-5}/0.0805 = 2.74 \times 10^{-4} \sim 99.97$ % ES (hemimercaptal-enzyme). E = apoenzyme; S = G3P; $K_S = [S][E]/[ES]$; K_M = Michaelis constant.

d. The anomalous rotatory dispersion is tentatively assumed to be due to Cotton effects. The origin of effect No. 1 is probably a Zn-complex.[28,29] The origin of No. 2 may be sulphur, since NAD binds near cystein.[4]

e. F = forward reaction: G3P → 3-PGA.

Table 2.

Protein	pH	[NAD]/[Enz.] Molar ratio	λ_c mμ[a]	$-K$[a]	r[b]	$-b_0$[c] Circ. dg.	Apparent helical content[c] %	No. of obs.
Apoenzyme	8.4	0	249.8 ± 1.7	13.0 ± 1.6	0.983	154 ± 6	24 ± 1	11
»	7.7	0	246.2 ± 1.1	13.6 ± 1.0	0.989	131 ± 6	21 ± 1	11
» α-thioglycerol	7.70	0	255.0 ± 0.2	11.00 ± 0.17	1.001	158 ± 6	25 ± 1	8
Holoenzyme (w. NAD) + »	7.70	9.2	253.8 ± 0.5	11.1 ± 0.4	1.004	158 ± 6	25 ± 1	9
» + »	7.70	22.5	252.6 ± 0.9	11.3 ± 1.4	0.950	158 ± 6	25 ± 1	7
Hemimercaptal-enzyme	7.70	0	242.9 ± 0.8	11.0 ± 1.1	0.972	98 ± 6	16 ± 1	11

a. λ_c and K were calculated by linear regression. Only the observations, which were considered to be free of significant perturbation due to the anomalous rotatory dispersion effects at 305 and 650 mμ, were used.

b. r = correlation coefficient.[22,25]

c. See text.

Table 3.

Comparison	Parameter	Significance of difference
Apoenzyme, pH 8.4 *vs.* pH 7.7	λ_c	+
	K	−
	b_0	+
Apoenzyme with α-thioglycerol *vs.* apoenz. without this compound; pH 7.7	λ_c	+
	K	+
	b_0	+
Holoenzyme, pH 7.7, [NAD]/[Enz.] = 9 *vs.* 23	λ_c	−
	K	−
	b_0	−
Apoenzyme, pH 7.7, w. α-thioglycerol *vs.* holoenz. w. 9 NAD/Enz., pH 7.7, w. α-thioglycerol	λ_c	+
	K	−
	b_0	+
Apoenzyme, pH 7.7 *vs.* hemimercaptalenz., pH 7.7	λ_c	+
	K	+
	b_0	+

proteins in a variety of solvents.[23] The dispersion of the refractive index was approximated with that of water.[22,25,30] The value of the parameter b_0 was calculated from a Moffitt plot[22] ($[m'](\lambda^2-\lambda_0^2)$ *vs.* $1/(\lambda^2-\lambda_0^2)$). The slope of the regression line is $= b_0\lambda_0^4$. The helical content was evaluated from the equation:

$$\% \text{ helix} = -b_0/630$$

The statistical treatment of the data was as previously described. In Table 3 an evaluation of the significance of the differences between the parameters of the proteins is made. The basis for the comparison is the standard error, Sy, and the relative error of the slope (Δ slope/slope).

DISCUSSION

An interpretation in details of optical rotatory dispersion data of proteins is difficult in the present state of our knowledge of the theory, but the method provides a very sensitive indicator of the occurrence of conformational changes

Table 4.

Protein	Range used for calculation of b_0 mμ
Apoenzyme at pH 8.4	320−500
» » pH 7.7	320−500
» + α-thioglycerol	325−400
Holoenzyme (w. NAD) + α-thioglycerol, m.r. 9.2	325−400
» » » » m.r. 22.5	325−400
Hemimercaptalenzyme	320−520

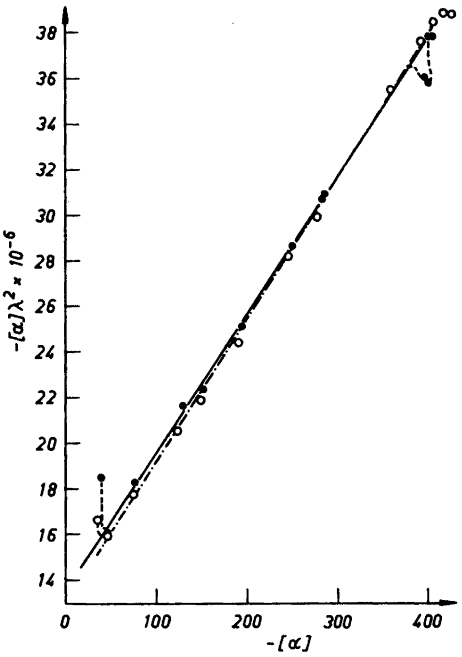


Fig. 1. Optical rotatory dispersion of glyceraldehyde-3-phosphate dehydrogenase apoenzyme in 0.15 M KCl, 0.001 M EDTA at 23.5°C plotted according to the one-term Drude equation omitting the points which are significantly influenced by the anomalous rotation near 305 and 650 mμ. See text. Symbols: O, pH 8.4, stippled line; ●, pH 7.7, full line. The lines were calculated by linear regression. Broken lines: Anomalous rotatory dispersion (probably Cotton effects).

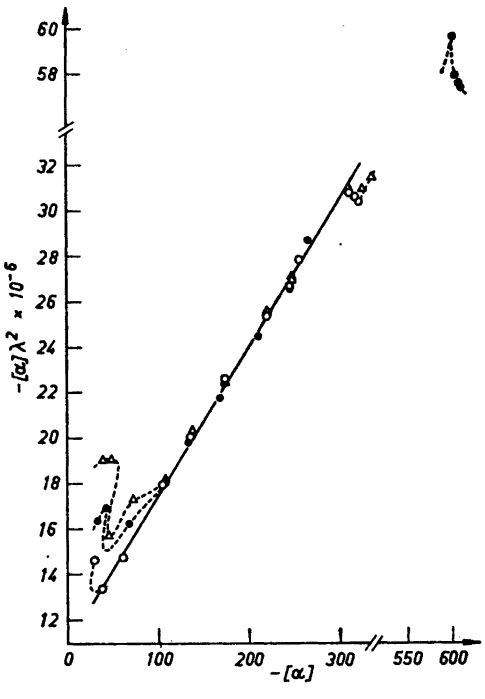


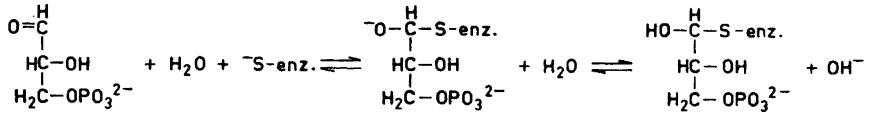
Fig. 2. Optical rotatory dispersion of the apoenzyme in 0.15 M KCl, 0.001 M EDTA, 6×10^{-4} M α-thioglycerol and various concentrations of NAD at pH 7.70 and 23°C. Plot: As in Fig. 1. Symbols: O: 0.0 [NAD]/[Enz.], molar ratio; ●: 9.2 m.r.; △: 23.0 m.r. Molecular weight of the protein: 140 000. The full line in the graph is the regression line for [NAD]/[Enz.] = 0. The broken lines indicate the contour of the anomalous rotatory dispersion.

in general. It is also possible to make a crude classification of the major changes into those representing alteration of the helical content and those involving the solvation of centres of asymmetry from the changes in b_0 (or λ_c) and a_0 (or K),[31] respectively. The location of the changes in the molecule, however, must be determined by a different method.

G3PDH is a protein with a relatively high helical content according to the estimates based on the amino acid composition [26,27] or on b_0. It is, therefore, not surprising that even a small pH-change alters the helical content significantly (Table 3 and Fig. 1). Addition of the non-polar solvent, α-thioglycerol, even at a low concentration, changes both the apparent helical content and the solvation of one or more of the centers of asymmetry (Table 3). The question of whether the effect of α-thioglycerol on the O.R.D. parameters is

specific or general remains to be answered. Binding of the stoichiometric quantity of NAD to the enzyme alters the apparent helical content, but not K. The presence of excess of NAD does not appear to give rise to further changes in the dispersion curve with the exception of an enhancement of the anomalous dispersion effect at 650 mμ (Table 3 and Fig. 2).

The formation of the enzyme-substrate intermediate in the forward reaction, G3P-hemimercaptal-enzyme, changes both the helical content and the symmetry (Table 3, Fig. 3). The following reaction scheme has been proposed:



The overall stability constant, $K_{stab} = [ES]/[E][S]$, is small (2.2×10^{-5} M). The measurements must therefore be made in the presence of a large excess of substrate.

Preliminary measurements of the optical rotatory dispersion parameters of the enzyme-substrate intermediate in the reverse reaction, 3-phosphoglyceric acid thiol-ester-enzyme, indicate that the parameters b_0, K, and λ_c are significantly different from those of the apoenzyme (at pH 7.7) and the hemimercaptal-enzyme. Thus, it appears that G3PDH also undergoes conformational changes accompanying its interaction with 1,3-diphospho-glyceric acid and that there exist at least two intermediates in the catalytic reaction, G3P-hemimercaptal-enzyme and 3-PGA-thiolester-enzyme, of individual conformation.

Further statements about the nature of the conformational changes probably require the application of more direct methods of structural analysis than optical rotatory dispersion.

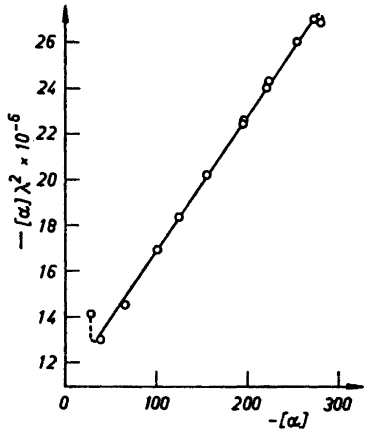


Fig. 3. Optical rotatory dispersion of the G3P-hemimercaptalenzyme in 0.15 M KCl, 0.001 M EDTA, 0.081 M G3P at pH 7.7 and 23.5°C. Line: Regression line.

Acknowledgements. This investigation was supported in part by a NATO Science Fellowship to the author allotted by the Danish Department of Education, in part by a grant from the National Institutes of Health to professor G. P. Hess, Cornell University. The economic assistance is gratefully acknowledged. The author also wishes to thank Dr. Hess for suggesting the project, professor H. A. Scheraga for the permission to use the spectropolarimeter, and Dr. E. Racker for the gift of the G3PDH and for advice.

REFERENCES

1. Labouesse, B., Havsteen, B. H. and Hess, G. P. *Proc. Natl. Acad. Sci. U.S.* **48** (1962) 2137.
2. Lumry, R. and Parker, H. *J. Am. Chem. Soc.* **85** (1963) 483.
3. Yagi, K., Ozawa, T. and Oii, T. *Biochim. Biophys. Acta* **77** (1963) 20.
4. Boyer, P. D. and Segal, H. L. In McElroy, W. D. and Glass, B. (Eds.) *The Mechanism of Enzyme Action*, Johns Hopkins Press, Baltimore 1954, p. 520.
5. Velick, S. F. and Furfine, C. In Boyer, P. D., Lardy, H. and Myrbäck, K. (Eds.) *The Enzymes*, 2nd Ed., Academic Press, New York 1963, Vol. 7, p. 243.
6. Krimsky, I. and Racker, E. *Science* **122** (1955) 319.
7. Krimsky, I. and Racker, E. *Biochem.* **2** (1963) 512.
8. Cori, G. T., Slein, M. W. and Cori, C. F. *J. Biol. Chem.* **173** (1948) 605.
9. Harris, I., Meriwether, B. P. and Park, J. H. *Nature* **198** (1963) 154.
10. Segal, H. L. and Gold, A. H. *J. Biol. Chem.* **238** (1963) PC 2589.
11. Hilvers, A. G., van Dam, K. and Slater, E. C. *Biochim. Biophys. Acta* **85** (1964) 206.
12. Monod, J., Changeux, J.-P. and Jacob, F. *J. Mol. Biol.* **6** (1963) 306.
13. Hvidt, Aa. and Kägi, J. H. R. *Compt. Rend. Trav. Lab. Carlsberg* **33** (1963) 497.
14. Boyer, P. D. In Benesch R. *et al.*, (Eds.) *Sulphur in Proteins*, Academic Press, New York 1959, p. 211.
15. Elödi, P. and Szabolcsi, G. *Nature* **184** (1959) 56.
16. Krebs, E. G. In Kaplan, N. O. and Colowick, S. P. (Eds.) *Methods of Enzymology*, 1st Ed., Academic Press, New York 1955, Vol. I, p. 410.
17. Fox, Jr., J. B. and Dandliker, W. B. *J. Biol. Chem.* **221** (1956) 1005.
18. Dandliker, W. B. and Fox, Jr., J. B. *J. Biol. Chem.* **214** (1955) 275.
19. Racker, E., Klybas, V. and Schramm, M. *J. Biol. Chem.* **234** (1959) 2510.
20. Bartlett, G. R. *J. Biol. Chem.* **234** (1959) 469.
21. Wu, R. and Racker, E. *J. Biol. Chem.* **234** (1959) 1029.
22. Havsteen, B. H. and Hess, G. P. *J. Am. Chem. Soc.* **85** (1963) 791.
23. Moffitt, W. *J. Chem. Phys.* **25** (1956) 467; Moffitt, W. *Proc. Natl. Acad. Sci. U.S.* **42** (1956) 736; Yang, J. T. and Doty, P. *J. Am. Chem. Soc.* **79** (1957) 761; Urnes, P. and Doty, P. *Advan. Protein Chem.* **16** (1961) 401.
24. Moffitt, W. and Yang, J. T. *Proc. Natl. Acad. Sci. U.S.* **42** (1956) 596; Shechter, E. and Blout, E. R. *Proc. Natl. Acad. Sci. U.S.* **51** (1964) 695, 794; Shechter, E., Carver, J. P. and Blout, E. R. *Ibid.* **51** (1964) 1029.
25. Havsteen, B. H. *Studies on Conformational Changes in α-Chymotrypsin accompanying its Catalytic Action* (Diss.) Cornell University, Ithaca, N. Y. 1962.
26. Davies, D. R. *J. Mol. Biol.* **9** (1964) 605.
27. Harris, J. J. and Perham, R. N. *Biochem. J.* **89** (1963) 60p.
28. Li, T.-K., Ulmer, D. D. and Vallee, B. L. *Biochem.* **2** (1963) 482.
29. Dixon, M. and Webb, E. C. *Enzymes*, Academic Press, New York 1958, p. 205.
30. Dorsey, N. E. *Properties of Ordinary Water Substance*, ACS Monograph Series, Reinhold Publ. Corp., New York 1940, p. 287.
31. Tanford, C., Buckley III, C. E., De, P. K. and Lively, E. P. *J. Biol. Chem.* **237** (1962) 1168; Tanford, C. *J. Am. Chem. Soc.* **84** (1962) 1747.

Received March 8, 1965.