[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, POLYTECHNIC INSTITUTE OF BROOKLYN]

Long-lived States in Photochemical Reactions. II. Photoreduction of Fluorescein and its Halogenated Derivatives[1,2]

BY ALBERT H. ADELMAN[3] AND GERALD OSTER

RECEIVED MARCH 22, 1956

A comparison is made among the constants characterizing the photoreduction of fluorescein and several of its halogenated derivatives in the presence of allyl thiourea. The quantum yield of production of the long-lived excited state changes little with halogenation. Similarly, the lifetimes of these states do not vary among the halogenated members and are about 10^{-4} sec. in water at room temperature. Internal conversion from the first singlet excited state to the ground state increases with halogenation. There is extensive quenching of the metastable state by the dye in the ground state as well as by a wide variety of foreign substances. The diversity of effective substances suggests that a diffusion-controlled quenching mechanism is operative as opposed to an energy transfer mechanism. Comparison of phosphorescence quenching in a highly viscous medium with the retardation of photoreduction indicates that the metastable state is the triplet state.

Introduction

In a previous paper[4] we showed that the photoreduction of eosin in the presence of allyl thiourea proceeds *via* a long-lived excited state of the dye molecule. The present work is concerned with the role of halogen substitution on the kinetics of photoreduction of fluorescein-type dyes. In particular, we are looking for the effect of such substitutions on the transition from excited singlet to long-lived excited states and on the lifetime of this long-lived state. All of the fluorescein dyes which we have studied exhibit the same behavior in the photoreduction as did eosin but differ in the values of the rate constants of the reactions. Data are presented which suggest that the long-lived state is the lowest triplet excited state of the dye molecule.

Experimental

Materials.—The dyes (fluorescein, 4',5'-dibromofluorescein, 4',5'-diiodofluorescein, 2',4',5',7'-tetrabromofluorescein, 2',4',5',7'-tetraiodofluorescein, 2',4',5',7'-tetrabromo-3,4,5,6-tetrachlorofluorescein, 2',4',5',7'-tetraiodo-3,4,5,6-tetrachlorofluorescein) were obtained either from Eastman Kodak Co. Organic Chemicals or from Allied Chemical and Dye Corporation. Hereafter, these dyes are referred to as Fl, $FlBr_2$, FlI_2, $FlBr_4$, FlI_4, $FlBr_4Cl_4$ and FlI_4Cl_4, respectively. The dyes were employed as the sodium salts. Those dyes obtained from Eastman (Fl, $FlBr_2$, FlI_2) were available only in the acid form so that in these cases the sodium salt was prepared. Allyl thiourea (Fisher, C.P. grade) was recrystallized from acetone. Prepurified nitrogen (Airco) was passed through finely divided hot copper to further remove traces of oxygen. The copper was periodically regenerated with hydrogen at elevated temperatures. Potassium iodide and *p*-phenylenediamine hydrochloride were Fisher C.P. grade and glycerol was Fisher Reagent grade. The photoreductions were carried out in $^1/_{15}$ *M* phosphate buffer at *p*H 7.0.

Procedure.—The fading of the dyes was followed with a recording colorimeter at a wave length corresponding to the absorption maximum of the dye, the same beam being used as both the actinic and analyzing radiation. The solutions were flushed with nitrogen prior to and during irradiation. The details as they apply to the fading and fluorescence quenching experiments have been presented in the previous paper.[4]

Only in the case of fluorescein at low reductant concentrations was the rate of fading too low to be measured when irradiated with necessarily low intensity monochromatic light. Quantum yields were determined with fluorescein (Fl) solutions containing higher concentration of reductant and employing monochromatic light. The rate data obtained with polychromatic light were then referred to those obtained with monochromatic light.

Aqueous solutions of *p*-phenylenediamine are rather rapidly oxidized, especially in the presence of these dyes in the light. Hence they were constantly flushed with nitrogen before addition to the reaction vessel. This procedure proved adequate to give reproducible results.

The absorption spectra of the dye solutions were determined in a Beckman model DU spectrophotometer. The fluorescence spectrum was obtained by using the irradiated sample as the light source, a Bausch and Lomb monochromator as the spectroscope, and a RCA 1P22 photomultiplier (S-8 response) as the detector. The output of the phototube was registered on a Leeds and Northrup Speedomax high impedance recorder whose time axis was synchronized with the movement of the wave length drum of the monochromator. The phototube was calibrated by comparing its response to the response of a thermopile at each wave length. This calibration was then converted from response to equal flux to response to equal numbers of quanta.[5]

Visual observations of the phosphorescence quenching of the dyes were made in glycerol at low temperatures. The solutions containing the reagents with the appropriate gases (nitrogen or oxygen) were cooled in liquid nitrogen. The glycerol glasses were excited with the 365 mμ lines of a mercury lamp and the phosphorescence was observed as the samples were warming.

Results

Spectra.—All of the dyes obey Beer's law at concentrations below 10^{-4} *M*. The absorption spectra show several maxima, the most prominent of which in the visible are given in Table I. Included also is the area under the molar absorbance (ϵ) *versus* wave number curve for the absorption band in the visible region. These dyes exhibit a shoulder on the short wave length side of the visible absorption band which becomes more prominent the greater the halogenation. In each case the area which we have chosen includes that under the shoulder.

The fluorescence spectra of the dyes remain unchanged irrespective of the wave length of the exciting light, at least for wave lengths in the visible region up to the absorption maximum. The absorption and fluorescence spectra of $FlBr_4Cl_4$ (phloxine B) is shown in Fig. 1. The fluorescence spectrum is an approximate mirror-image of the absorption spectrum including the shoulder. The fluorescence spectra shift to longer wave lengths as the halogenation is increased in the same manner as do the visible light absorption spectra. Similarly, the β-phosphorescence in rigid media shift to longer wave lengths.

(1) Presented before the Division of Physical and Inorganic Chemistry at the 129th A.C.S. Meeting in Dallas, April, 1956.

(2) This paper represents a part of the dissertation submitted by A. H. Adelman to the faculty of the Graduate School of the Polytechnic Institute of Brooklyn in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

(3) Eastman Kodak Fellow, 1955–1956.

(4) G. Oster and A. H. Adelman, THIS JOURNAL, **78**, 913 (1956).

(5) Mr. Neil Wotherspoon designed the method and constructed the apparatus. Details are contained in his forthcoming Ph.D. thesis.

TABLE I

ABSORPTION CHARACTERISTICS OF FLUORESCEIN AND ITS HALOGENATED DERIVATIVES

Dye	Absorption max ($m\mu$)	$\epsilon_{max} \times 10^{-4}$ (cm.$^{-1}$ mole^{-1})	$\int \epsilon d(1/\lambda) \times 10^{-8}$ (cm.$^{-2}$ mole^{-1})
Fl	490	5.46	1.30
$FlBr_2$	506	5.46	1.13
FlI_2	510	5.64	1.09
$FlBr_4$	519	8.20	1.35
FlI_4	528	8.76	1.42
$FlBr_4Cl_4$	540	7.60	1.21
FlI_4Cl_4	550	8.15	1.34

The intensity of fluorescence is not correlated simply with the extent of halogenation; the intensity of fluorescence (determined visually) decreases in the following order Fl (0.71), $FlBr_2$, $FlBr_4$ (0.15), $FlBr_4Cl_4$, FlI_2, FlI_4, FlI_4Cl_4 (0.02) where the figures in parentheses refer to the fluorescence yields quoted by Pringsheim.[6]

Fluorescence and Phosphorescence Quenching.—The fluorescence of all the dyes can be quenched by potassium iodide, allyl thiourea and *p*-phenylenediamine. The quenching follows the Stern–Volmer relation with the quenching constants given in Table II. We observed that the absorption spectra of the dyes remain unchanged upon the addition of quencher while the depolarization of fluorescence changed indicating that the quenching is diffusion-controlled.

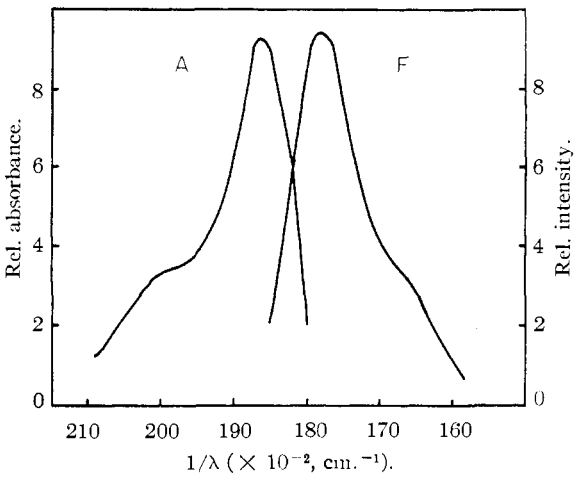

Fig. 1.—Absorption and fluorescence spectra of phloxine B: A, absorption spectrum; F, fluorescence spectrum.

TABLE II

STERN–VOLMER FLUORESCENCE QUENCHING CONSTANTS (liter/mole)

Quencher dye	Fl	$FlBr_2$	FlI_2	$FlBr_4$	FlI_4	$FlBr_4$-Cl_4	FlI_4-Cl_4
Potassium iodide	9.6	6.3	3.8	4.0	3.1	3.0	3.4
Allyl thiourea	15	12	8.2	13	5.2	11	8.4
p-Phenylenediamine	54	44	29	43	32	28	22

Fluorescein at a concentration of 10^{-5} M in glycerol exhibits a yellowish β-phosphorescence. Visual observations of the phosphorescence in the neighborhood of $-100°$ show that *p*-phenylenediamine at 10^{-4} M is a powerful quencher, oxygen is weaker, while potassium iodide and allyl thiourea are ineffective at this concentration. However, at higher concentrations (10^{-3} M) of potassium iodide and allyl thiourea quenching is observed. No quenching of phosphorescence is observed with any of the solutions at very low temperatures ($-180°$), while at higher temperatures ($-79°$) no phosphorescence is observable by eye, hence an intermediate temperature was chosen.

Photoreduction.—As with eosin, the following kinetic scheme is consistent with the fading data for all of the dyes[4]: (1) D + $h\nu$ → D*, (2) D* → D, (4) D* + A → D + A, (5) D* → D′, (6) D′ + A → Products, (7) D′ → D, (8) D′ + D → D + D, (9) D′ + X → D + X. Where D is the unexcited dye, D* is the dye in the first excited singlet state, D′ is the dye in the long-lived excited state, A is the reductant (allyl thiourea), and X is a substance (for example *p*-phenylenediamine) which retards the reaction by causing D′ to go to the ground state. Step 1 is normal excitation and step 2 is the transition (including fluorescence) of the excited singlet state to the ground state. Step 4 is the fluorescence quenching by allyl thiourea but is important only at reductant concentrations higher than those which we have employed. Step 5 is the transition to the metastable state D′ which reacts with the reductant to form products (step 6). The long-lived species can undergo transition to the ground state with or without emission (step 7). Deactivation of the metastable state can occur also *via* interaction with unexcited dye or with some foreign molecule (steps 8 and 9).

Applying the usual steady-state assumption to the transient species D and D′ one obtains the following expression of the initial rate

$$Ik_5k_6(A)/\{(k_2 + k_5)[k_7 + k_6(A) + k_8(D)]\}$$

where I is the intensity of the light absorbed and where the k's are the rate constants for the reactions indicated by the subscripts. Figure 2 is an example of the applicability of this expression to our data. The intercept, namely, the quantum yield at infinite reductant concentration, Φ_{max} is the quantum yield for the formation of the metastable state. The slopes of the lines gives Φ_{max}^{-1} $[k_7/k_6 + k_8(D)/k_6]$.

Certain substances, such as *p*-phenylenediamine (PPD), retard the photoreduction by attacking the metastable state (step 9) preferentially because this state is longer lived. The ratio of rates without, R_0, and with, R, this retarder is given by $R_0/R = 1 + [k_9(X)]/[k_7 + k_8(D) + k_6(A)]$. We found that for all the dyes this ratio is experimentally a linear function of the concentration of the retarder (X), so that the postulated mechanism is consistent with the data. From the slope of such a plot and the data for the unretarded reaction, we obtain values for k_9/k_7. This is the Stern-Volmer constant for the metastable state. With potassium iodide, however, the deactivation of the excited singlet state is comparable with that for the metastable state. In this case, the ratio R_0/R is calculated to be quadratically dependent

(6) P. Pringsheim, "Fluorescence and Phosphorescence," Interscience Publishers, Inc., New York, N. Y., 1949, p. 316.

TABLE III

CONSTANTS DERIVED FROM THE PHOTOREDUCTION

	Fl	$FlBr_2$	FlI_2	$FlBr_2$	FlI_4	$FlBr_4Cl_4$	FlI_4Cl_4
Φ_{max} ($\times 10^2$)	2.01	12.4	3.4	9.2	4.7	15.0	3.6
k_6/k_7 (l./mole)	35	81	25.8	47.4	44.3	44.3	118
k_8/k_7 ($\times 10^{-3}$) (l./mole)	15.1	91.6	100	48.7	97	108	97
PPD k_9/k_7 ($\times 10^{-4}$) (l./mole)	10.6	82.4	23.2	60.8	72.5	142	76
KI k_9/k_7 ($\times 10^{-3}$) (l./mole)	7.15	4.65	1.48	2.22	0.70	9.65	1.02

on the potassium iodide concentration. Again this is experimentally verified for all the dyes.

In Table II are given the experimentally obtained constants for all the dyes studied.

Discussion

The shoulder in the visible light absorption spectrum of phloxine (Fig. 1) cannot be ascribed to dimer formation[7] since it appears in the concentration range where Beer's law is obeyed and also appears in the "mirror-image" fluorescence spectrum. Hence we have included the shoulder in the evaluation of the area under the absorption curves. With increasing halogenation the absorption maxima shift to longer wave lengths. Empirically, the wave length maximum λ_{max} of these dyes is a simple function of the extent of halogenation, namely, $\lambda_{max} = \lambda_{max}\,(Fl) + 1.4 \times 10^{24} \sum_i \alpha_i$ where the wave lengths are in $m\mu$ and α_i are polarizabilities (in cm.^{3}) of the free halogen ions taken from Pauling.[8] Although the energy levels change in a regular way with halogenation, the oscillator strengths[9] (proportional to areas under the curves, Table I) do not change in a regular way and are equal to $f = 0.54 \pm 0.07$. Similarly, the intrinsic lifetimes of the first excited states are effectively the same for all the dyes, namely, $\tau_0 = 4.4 \pm 0.6 \times 10^{-9}$ sec.

With increasing halogenation the fluorescence yield of the dyes decreases markedly. This decrease in fluorescence is a consequence of some competing transition of the excited singlet to either the ground state or, as Kasha[10] has suggested, transition to the triplet excited state. Our values of Φ_{max} (Table III) give the fraction of the excited molecules which undergo transition to the metastable state. For the halogenated derivatives of fluorescein this does not increase in a regular way with increasing halogenation. Hence it is the internal conversion from the excited singlet state to the ground state which must increase with halogenation. For example, knowing Φ_{max} and the fluorescence yield of fluorescein (Fl), eosin ($FlBr_4$) and erythrosin (FlI_4), we calculate that the fraction of singlet excited molecules which undergo radiationless transitions directly to the ground state are 0.17, 0.36 and 0.93, respectively.

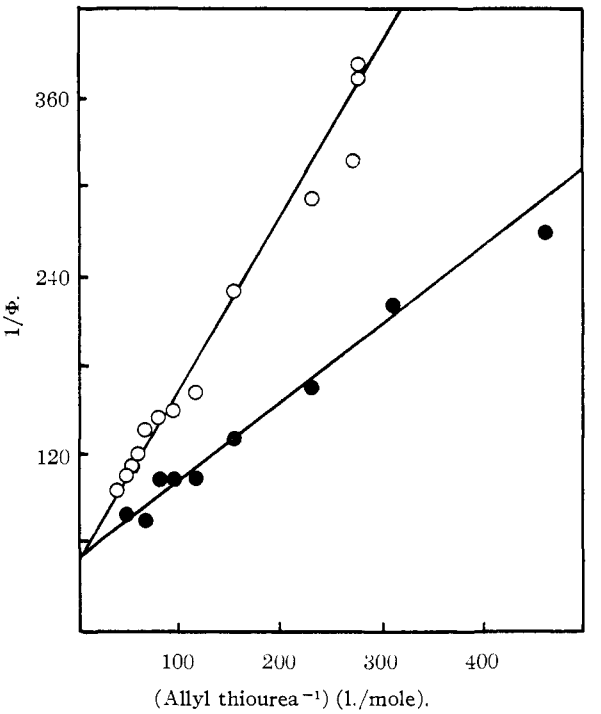

Fig. 2.—Quantum yield of the photoreduction of fluorescein; dye concentration: ●, 0.64×10^{-5} M; ○, 1.92×10^{-5} M.

The fluorescence lifetime of some of these dyes have been measured. For Fl, FlB_4 and FlI_4, they are 4.5, 1.9 and 1.0×10^{-9} sec., respectively.[11] The fluorescence quenching constants (Table II) decrease in a parallel manner. The relative quenching efficiencies among three quenchers remains about the same for all the dyes. For example, in all cases *p*-phenylenediamine is about ten times more efficient than is potassium iodide. Apparently the mechanism of the diffusion-controlled quenching is the same for all these quenchers but they differ only in the magnitude of their quenching ability.

p-Phenylenediamine and many other substances, particularly picric acid, aniline, tartrazine, riboflavine, 4,4′-diaminostilbene-2,2′-disulfonic acid, fluorescein (Fl) and dibromofluorescein ($FlBr_2$), were most efficient and equally effective as retarders for the photoreduction of phloxine ($FlBr_4Cl_4$). This suggests that all these diverse substances are retarding at optimal efficiency and hence we can estimate the lifetime of the metastable state. Taking k_9/k_7 to be 10^6 liters per mole as a repre-

(7) Compare, B. Soederberg, *Ann. Physik*, **41**, 381 (1913).

(8) L. Pauling, *Proc. Roy. Soc. (London)*, **A114**, 191 (1927).

(9) The oscillator strength f and the intrinsic lifetime τ_0 are given by the formulas [R. Ladenburg, *Z. Physik*, **4**, 451 (1921), R. Ladenburg and F. Reiche, *Naturwiss.*, **11**, 584 (1923)] $f = 4.32 \times 10^{-9} \int nK^2 ed(1/\lambda)$ and $1/\tau_0 = 2.88 \times 10^{-9}\,(n/\lambda_{max})^2 \int nK^2 ed(1/\lambda)$ where n, the refractive index of the medium, is applied to correct for the wave length in the medium. In a medium of refractive index n the local field differs from that of the applied field by the factor $K = (2n^2 + 1)/3n^2$ (G. Oster, *Bull. soc. chim.*, D322 (1949)) and not, as is commonly supposed, by the Lorentz factor (compare, W. Kuhn, *Z. physik. Chem.*, **B30**, 356 (1935)). For water $nK^2 = 1.0$.

(10) M. Kasha, *Disc. Faraday Soc.*, No. 9, 14 (1950). See also R. S. Becker and M. Kasha in "The Luminescence of Biological Systems," edited by F. H. Johnson, American Association for the Advancement of Science, Washington, D. C., 1955, pp. 25–42.

(11) E. Gaviola, *Z. Physik*, **42**, 853 (1927).

sentative value, and since there are 6.6×10^9 diffusion-controlled encounters per sec. in a liter of molar solution at room temperature in water, then if each encounter is effective (*i.e.*, optimal efficiency) the lifetime will be about 10^{-4} sec. The constancy, within a factor of two of the retardation constants of the halogenated members suggests that the lifetimes of the metastable states of such molecules changes very little with the nature or the extent of the halogenation. Some direct measurements of phosphorescence lifetimes of halogenated benzenes[12,13] in rigid media indicate that the lifetimes are practically independent of the extent of halogenation, although a decreased lifetime is obtained when chlorine is replaced with bromine. For the best retarders Fl shows a markedly lower retardation constant than the other dyes, indicative of a shorter lifetime. On the other hand, visual comparison of the phosphorescences of fluorescein (Fl) and eosin (FBr_4) in glycerol at $-180°$ indicates that the latter dye has the shorter lifetime. This may be explained on the basis of the lifetimes of the halogenated dyes changing less rapidly with viscosity[14] than that of fluorescein because of a protecting effect of the halogens. This protecting effect (fewer non-radiative transitions with increasing halogenation) has been noted by McClure, *et al.*,[12] for the bromobenzenes.

The retardation by the ground state dye (step 8), by other dyes of the family, and by the other foreign materials noted above is not correlated with the absorption and emission properties of the dye being faded or the retarder, but can be explained simply by a diffusion-controlled quenching mechanism. Mechanisms of quenching based on intermolecular energy transfer[15] are probably unlikely here since the retarders have a diversity of absorption and emission characteristics. Further, substances having these characteristics in common show widely dissimilar effects (*e.g.*, 4,4′-diaminostilbene-2,2′-disulfonic acid is effective while quinine sulfate is not; aniline is effective but bromobenzene is not, etc.).

Lewis, *et al.*,[16] have identified the β-phosphorescence of fluorescein as arising from the transition from the lowest triplet level of the dye to its ground state. It is not unlikely that this is also the origin of the β-phosphorescence of the halogenated fluorescein derivatives. Our phosphorescence quenching experiments reported above in which there is a parallelism between the efficiencies of retardation and of phosphorescence quenching[17] indicates strongly that the metastable species deduced from the kinetic data on the photoreduction is indeed the triplet state of the dyes.

Acknowledgments.—This work was supported by the Photographic Branch of the Signal Corps (Contract No. DA-36-039 sc-42463) and by the Air Research and Development Command of the Air Corps (Contract No. AF 18(600)-1182).

(12) P. P. Dikun, A. A. Petrov and B. Ya. Svishnikov, *Zhur. Eksptl. Teoret Fiz.*, **21**, 150 (1951).

(13) D. S. McClure, N. W. Blake and P. L. Hanst, *J. Chem. Phys.*, **22**, 255 (1954).

(14) Compare, B. Ya. Sveshnikov and P. P. Dikun, *Doklady Akad. Nauk, S.S.S.R.*, **60**, 371 (1948). See also G. Porter and M. W. Windsor, *Disc. Faraday Soc.*, No. 17, 178 (1954).

(15) See T. Förster, *Ann. Physik*, **2**, 55 (1948).

(16) G. N. Lewis and M. Kasha, THIS JOURNAL, **66**, 2100 (1944); G. N. Lewis and M. Calvin, *ibid.*, **67**, 1232 (1945); for review, see M. Kasha, *Chem. Revs.*, **41**, 401 (1947).

(17) Compare, S. Boudin, *J. chim. phys.*, **27**, 285 (1930); H. Kautsky and A. Hirsch, *Chem. Ber.*, **64**, 2677 (1931).

BROOKLYN, N. Y.

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Conversion of Fibrinogen to Fibrin. XIX. The Structure of the Intermediate Polymer of Fibrinogen Formed in Alkaline Solutions[1]

BY EDWARD F. CASASSA

RECEIVED JANUARY 3, 1956

The enzymatic action of thrombin results in a high degree of conversion of bovine fibrinogen to a soluble polymer in a solvent of *p*H 9.5 and molar ionic strength 0.45, but fibrin gel does not appear provided the protein concentration is sufficiently low. Conventional treatment of light scattering data from the polymer solutions, by extrapolation of the intensity to zero scattering angle, is shown to lead to meaningless values of molecular weight and radius of gyration. However, the experimental results do conform to the scattering behavior of thin rod-like particles for the asymptotic limit of indefinitely great length; and it is possible to determine the ratio of molecular mass to length though neither can be determined separately. Comparison of this ratio with that of monomeric fibrinogen leads to the conclusion that the polymer cross-section is double that of the monomer.

Introduction

When acted upon by thrombin, the plasma protein fibrinogen polymerizes to form fibrin, a three-dimensional network structure. The process comprises several steps, reversible at least under some conditions, as shown by the scheme[2,3]

$$F \underset{}{\overset{th}{\rightleftharpoons}} f \rightleftharpoons f_n \rightleftharpoons \text{fibrin}$$

An enymzatic reaction with thrombin th converts fibrinogen F to an activated form f; an intermediate polymer f_n then appears and this participates in the fibrin gel formation. The rates and equi-

(1) This investigation was supported by the Office of Naval Research, United States Navy, under Contract N7onr-28509.

(2) In previous publications from this Laboratory[3] the first and third steps of the reaction sequence were not indicated as reversible. Recent work of Donnelly, Laskowski, Notley and Scheraga,[4] however, has demonstrated the reversibility of these processes.

(3) J. D. Ferry, *Proc. Nat. Acad. Sci.*, **38**, 566 (1952).

(4) T. H. Donnelly, M. Laskowski, Jr., N. Notley and H. A. Scheraga, *Arch. Biochem. Biophys.*, **56**, 369 (1955); T. H. Donnelly, M. Laskowski, Jr., and H. A. Scheraga, paper presented at the 128th meeting of the American Chemical Society, Minneapolis, Minnesota, September, 1955.