only true if the interaction is strong. For weak or intermediate coupling we must consider the shift of each vibrational level and substitute in eq. A15 the much smaller oscillator strength for a single vibronic transition. This causes the calculated shift in energy to be negligible. The criterion[23] for weak or intermediate coupling is that the calculated energy shift, eq. A16, be less than the band width of the absorption. For DNA polymer or monomers the width of the band at half maximum is about 6500 cm.$^{-1}$. This is larger than the values obtained from eq. A16, and therefore it is consistent with the absence of a significant shift in the absorption maximum.

Acknowledgments.—We would like to thank Professor W. T. Simpson, University of Washington, for many helpful discussions and suggestions about the theory of hypochromism. He made the suggestion of weak coupling to explain the lack of shift in wave length maximum. Thanks are due Professor C. A. Dekker, Dr. A. Pour-El and Mr. H. Barrett, who stimulated the author's interest in this problem and furnished some DNA for experiments. Mr. Barrett performed the experiment on heated DNA. Dr. R. Haselkorn kindly made available a copy of his thesis. Professor P. Doty, Harvard, and Professor M. Kasha, Florida State, carefully read the manuscript and made valuable suggestions. We greatly appreciate their help.

[CONTRIBUTION FROM THE DIVISION OF PROTEIN CHEMISTRY, C.S.I.R.O., MELBOURNE, AUSTRALIA, AND THE DEPARTMENT OF CHEMISTRY, CORNELL UNIVERSITY, ITHACA, NEW YORK]

Effect of Light Scattering on Ultraviolet Difference Spectra[1]

BY S. J. LEACH AND H. A. SCHERAGA

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Ultraviolet difference spectra obtained with protein solutions may contain a contribution not only from a shift in the spectrum of the tyrosyl group but also from Rayleigh light scattering when there are large differences in the state of molecular aggregation. The scattering contribution may be subtracted out by extending spectral measurements into the visible region. When this procedure is used for insulin solutions, anomalies near the *p*H region of insolubility disappear and the difference spectra show a *p*H dependence which is consistent with the existence of a tyrosyl–carboxylate ion hydrogen bond in which the carboxylate acceptor group has an observed pK of 3.5 to 3.6.

Introduction

Previous work on the *p*H-dependence of the ultraviolet difference spectrum of insulin[2] indicated an apparently anomalous increase in the optical density in the *p*H regions 3.5 to 4 and 7.0 to 8.0 (see Fig. 6 of ref. 2). Furthermore, in this *p*H range optical density differences were finite though small even at say 310 mμ, where insulin solutions are not expected to show light absorption. It seemed possible that large molecular aggregates, present in solution near the region of insolubility (*p*H 4 to 7), gave rise to an increase in the optical density due to light scattering. The present work was therefore carried out to assess the influence of light scattering on the *p*H-dependence of the ultraviolet difference spectrum in a solution where the state of aggregation varies markedly with *p*H.

Experimental

All materials (including Eli Lilly crystalline beef zinc insulin, batch No. 535, 664), solutions, method of measurement, etc., were similar to those previously described.[2] The only difference was that a Beckman model DK-2 ratio-recording spectrophotometer was used instead of the model DU. The concentration of the insulin solutions was 0.5% and matched silica cells of 0.5 cm. light path were used. Owing to the high optical densities of these solutions it was necessary to use the photomultiplier, and slit-widths did not exceed 0.71. For comparison with previous results,[2] the data were in some cases converted to a standard basis for a 1 cm. cell. Insulin solutions at *p*H 1.5 were used as the reference and the optical densities, ΔD, of matched solutions[2] at higher *p*H's were determined over the wave length range of 240–600 mμ with a hydrogen lamp source for the whole range.

(1) This investigation was supported, in part, by Research Grant G-6461 from the National Science Foundation.

(2) M. Laskowski, Jr., S. J. Leach and H. A. Scheraga, THIS JOURNAL, **82**, 571 (1960).

Theory

The value of ΔD, due to different absorbances at each *p*H, may be augmented by a contribution from light scattering if the state of aggregation is different from that at the reference *p*H. The light scattering effect may be represented in terms of the turbidity τ by the approximate equation at finite concentration

$$\frac{I_t}{I_0} = e^{-\tau x} \qquad (1)$$

where I_t/I_0 is the fraction of the light transmitted and x is the length of the scattering medium, taken here as 1 cm. The quantity τ may be related to the molecular weight M and concentration c (in g./cc.) of the solute by equation 2, which is an approximation at finite concentration.

$$\tau = HcM \qquad (2)$$

where

$$H = \frac{32\pi^3}{3N\lambda^4} n_0^2 \left(\frac{dn}{dc}\right)^2 \qquad (3)$$

and λ is the wave length in cm., N is Avogadro's number, n_0 is the index of refraction of the solvent and dn/dc is the refractive increment. From eqs. 1 and 2 the transmittances at two *p*H's, say 1.5 and 3.5, may be related.

$$\frac{(I_t/I_0)_{pH\ 1.5}}{(I_t/I_0)_{pH\ 3.5}} = \frac{(e^{-HcM})_{pH\ 1.5}}{(e^{-HcM})_{pH\ 3.5}} \qquad (4)$$

Hence, defining ΔD_{286} for scattering as

$$\Delta D_{286} = \log\left(\frac{I_0}{I_t}\right)_{pH\ 3.5} - \log\left(\frac{I_0}{I_t}\right)_{pH\ 1.5} \qquad (5)$$

we obtain for the scattering contribution to ΔD_{286}

$$\Delta D_{286} = Hc\,(M_{pH\ 3.5} - M_{pH\ 1.5})/2.303 = Hc\Delta M/2.303 \qquad (6)$$

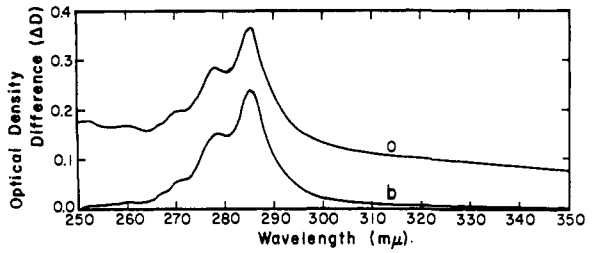

Fig. 1.—Difference spectra for 0.5% beef zinc insulin at *p*H 3.82 relative to a solution at *p*H 1.50. Measurements were made in matched 0.5 cm. cells up to 600 mμ but results are calculated for 1.0 cm. cells and shown only to 350 mμ: a, experimental curve; b, corrected as in text for scattering contribution.

Ignoring possible complications arising from the fact that the above theory is being applied to a region of absorption and making judicious estimates[3] of the values of $(dn/dc)_{286}$ and ΔM, it seems that one might account for the anomaly of the previous paper[2] in terms of a scattering contribution.

However, in view of the fact that such estimates are only approximate, it was desirable to obtain the scattering contribution directly from experiment. For this purpose, the above equations for the scattering contribution may be re-cast in the form

$$\Delta D_{286} = k\lambda^{-n} \qquad (7)$$

where k and n are constants. According to equation 7, a log–log plot of the experimental data should be linear in the wave length region where the effect is due to scattering and should depart from linearity in the wave length region of absorption. Extrapolation of the linear portion due to scattering into the absorption region indicates the scattering contribution, which may be subtracted from the total optical density in order to obtain the absorption effect.[4]

Results and Discussion

A typical experimental difference spectrum is shown in Fig. 1a for a pair of insulin solutions of *p*H 1.50 and 3.82, respectively. It should be noted that the curve does not return to the baseline either at 250 or 310 mμ because of the scattering contribution, insulin being more highly aggregated at *p*H 3.82 than at 1.50.[5] The data of Fig. 1a are plotted on a log–log scale in Fig. 2a. In the long wave length region of the visible part of the spectrum, *viz.* 350–600 mμ, where the protein does not absorb, the total (linear) optical density may be assumed to arise solely from light scattering. This linear portion with a slope[6] n of 2.5

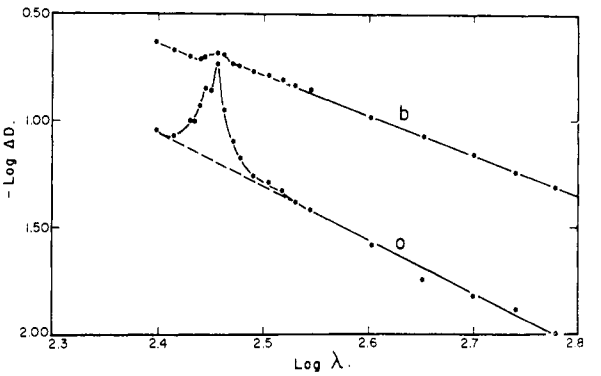

Fig. 2.—Double logarithmic plot of difference spectra for 0.5% beef zinc insulin, measured in 0.5 cm. cells: a, *p*H 3.82 relative to *p*H 1.50, slope = 2.5; b, *p*H 3.78 relative to *p*H 3.60, slope = 1.9.

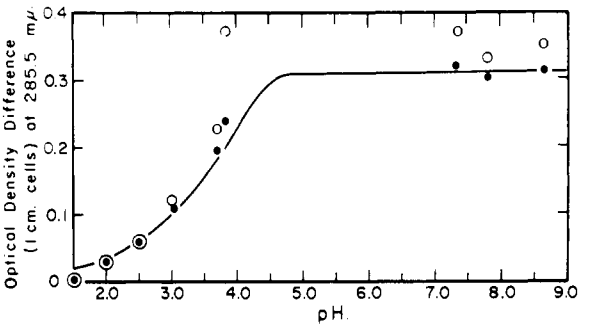

Fig. 3.—*p*H dependence of $\Delta D_{285.5}$ for 0.5% beef zinc insulin relative to a solution at *p*H 1.50. Measurements in 0.5 cm. cells are calculated for 1.0 cm. cells: ○, experimental points; ●, points corrected for ΔD contribution due to light scattering. The curve is the experimental titration curve of Tanford and Epstein[9] for the COOH ionization range of zinc insulin.

may be extrapolated to shorter wave lengths, as shown in Fig. 2a and the scattering contribution subtracted from the experimental curve. The resulting data, due to absorption, are shown as a ΔD *vs.* λ plot in Fig. 1b. It may be noted that the curve of Fig. 1b now has a baseline at $\Delta D = 0$ in the non-absorbing region.

The large contribution from scattering near the *p*H region of insolubility may be demonstrated with the data of Fig. 2b. Here, the pair of solutions was at *p*H 3.60 and 3.78, respectively. In this curve 90% of the optical density difference is due to scattering[7] and n is 1.9.

The experimental values of ΔD at the peak

(3) A *very rough* estimate of the light scattering contribution was made as follows: the value of $(dn/dc)_{286}$ was taken as 0.29 on the basis of a superposition of the effect of anomalous dispersion (estimated from data on benzene) on the normal dispersion of dn/dc for insulin. The resulting value of H, together with values of $c = 5 \times 10^{-3}$ g./cc. and $\Delta M = 100{,}000$ between *p*H 3.5 and *p*H 1.5, gives $\Delta D_{286} = 0.03$ from equation 6. This is about one-half of the size of the scattering anomaly in Fig. 6 of ref. 2.

(4) E. G. Bendit, private communication. See also P. Doty and E. P. Geiduschek, in "The Proteins," Vol. IA, Ed. by H. Neurath and K. Bailey, Academic Press, Inc., New York, N. Y., 1953, p. 393.

(5) P. Doty, M. Gellert and B. Rabinovitch, THIS JOURNAL, **74**, 2065 (1952).

(6) Since we have no information about the size of the aggregates formed, it is not possible to interpret the numerical value of n at present. Probably, the departure of n from the value of 4 indicates that the particles are very large. This is consistent with the large contribution of scattering which is $\Delta D = 0.13$ at *p*H 3.8.

(7) One might question the use of equation 7 when, as in Fig. 2, the linear portion is extrapolated into the absorbing region of the spectrum. Aside fron the internal consistency of the data (*e.g.*, $\Delta D = 0$ in the non-absorbing region), the following evidence suggests that this procedure is valid for the case under consideration here: the curve of Fig. 2b seems to correspond primarily to scattering, as it should for a *p*H 3.78 *vs.* *p*H 3.60 pair of solutions which presumably differ primarily only in the state of aggregation of the protein and not in the state of ionization of the group perturbing the chromophore. For further justification of the validity of this procedure also see ref. 8.

(8) E. Schauenstein and H. Bayzer, *J. Polymer Sci.*, **16**, 45 (1955).

wave length of 285.5 mμ may therefore be corrected for the scattering contribution at every *p*H. The values of $\Delta D_{285.5}$ arising solely from absorption may then be plotted as a function of *p*H, as shown in Fig. 3. The points fit reasonably well to the curve representing the experimental titration curve for insulin in the acid *p*H range.[9] It is important to use the experimental rather than a theoretical titration curve for acid groups since the calculation of the electrostatic factor wZ is subject to considerable uncertainty.[9] On the other hand, the use of the insulin titration curve is not entirely satisfactory since the curve represents ionizations of 6 carboxyl groups of intrinsic pK 3.6 and 4.7, while the spectral data probably refer, as pointed out in a previous paper,[2] to only one or possibly two ionizing groups. Having demonstrated the effect of light scattering on the ultraviolet difference spectrum, the anomalies near the insolubility region previously reported[2] disappear, and the significance of the titration curve of Fig. 3 remains as discussed previously, *i.e.*, the data are compatible with the presence of a tyrosyl-carboxylate ion hydrogen bond in which the carboxylate acceptor has an observed pK of 3.5 to 3.6.

In conclusion, whereas ultraviolet difference spectra may provide information on internal hydrogen bonding in non-aggregating systems, a possible contribution from light scattering must be taken into account if the state of aggregation of the protein is *p*H-dependent.

(9) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2170 (1954).

[CONTRIBUTION FROM THE BALLISTIC RESEARCH LABORATORIES, ABERDEEN PROVING GROUND, MARYLAND]

The Reaction of Nitric Oxide with Triethyl Phosphite

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The liquid phase reaction, $2NO + (EtO)_3P \rightarrow N_2O + (EtO)_3PO$, is described. The reaction is first order in nitric oxide and first order in phosphite and the reaction rate is quite insensitive to the nature of the solvent. A mechanism is proposed for the reaction which involves the intermediate $(EtO)_3PNO$.

A reaction which does not appear to have been previously reported in the literature has been found to occur between nitric oxide and triethyl phosphite to yield nitrous oxide and triethyl phosphate

$$2NO + (C_2H_5O)_3P \longrightarrow N_2O + (C_2H_5O)_3PO$$

The reaction goes cleanly and quickly in the liquid phase at room temperature. When the reaction is run in the presence of excess phosphite, the nitrous oxide that is formed shows no tendency to be further reduced to nitrogen. In the succeeding paragraphs, we present a study of the kinetics of the reaction and a mechanism which is consistent with the kinetic data. After the completion of the experimental portion of this work, a paper appeared by Cox and Westheimer,[1] which describes a reaction between nitrogen dioxide and phosphite esters to give the corresponding phosphate ester and nitrous oxide and nitrogen. This reaction is said to proceed rapidly, even at −80°, and has proved useful[2] for the preparation of phosphate esters, particularly in instances where the phosphate ester is sensitive to acid or base. Since nitric oxide is much less reactive than nitrogen dioxide, it would appear that the reaction described here is also of preparative value. It also constitutes a convenient method of preparing very pure nitrous oxide.[3]

The products and stoichiometry of the reaction were determined by stirring triethyl phosphite (0.1 mole) at room temperature in a system containing nitric oxide at 300 mm. (0.036 mole). The final pressure at the end of the reaction was 144 mm. (slightly less than the theoretical 150 mm. due to the greater solubility of nitrous oxide in the liquid as compared with nitric oxide). The gas was found by both infrared and mass spectrometric analysis to be nitrous oxide of purity greater than 99%. Gas chromatography of the liquid after reaction showed that it consisted of triethyl phosphite and triethyl phosphate, the yield of the latter being 0.018 mole (100%).

For the kinetic studies the reaction was carried out by rapidly stirring a solution of triethyl phosphite in the desired solvent in an atmosphere of nitric oxide. The rates were followed manometrically. Because the reaction is quite rapid, very vigorous stirring is required to obtain conditions such that the reaction rate is independent of stirring speed. By using a high-speed motor and a stirrer of the proper shape (see Experimental), it was possible to make the reaction rate independent of stirring speed as shown by the fact that in a series of experiments in which the initial concentration of reactants was kept constant but the stirring speed was varied, the observed reaction rate was constant. The phosphite was generally present in considerable excess, the initial molar ratio of phosphite to nitric oxide being about 4, so that the concentration of phosphite did not change by more than 10% during the first 60% of the reaction. Under these conditions good first order rate curves were obtained, as shown in Fig. 1.

The results from a number of runs at 0° using different initial concentrations of phosphite, different initial pressures of nitric oxide and different solvents are given in Table I. When the initial

(1) J. R. Cox, Jr., and F. H. Westheimer, THIS JOURNAL, **80**, 5441 (1958).

(2) J. R. Cox, Jr., Robert Wall and F. H. Westheimer, *Chem. and Ind. (London)*, 929 (1959).

(3) In the paper by Cox and Westheimer (ref. 1), the statement is made that phosphite esters do not react with nitric oxide. In response to private communication with Professor Westheimer, further experiments by Cox and Westheimer have, however, fully confirmed the results here reported.