

LIGHT SCATTERING INVESTIGATION OF ORDERING EFFECTS IN SILICOTUNGSTIC ACID SOLUTIONS¹

BY M. J. KRONMAN AND SERGE N. TIMASHEFF

Eastern Regional Research Laboratory,² Philadelphia 18, Pennsylvania

A light scattering investigation was carried out on silicotungstic acid in the presence and absence of salt. In the presence of 0.300 M NaCl, the dependence of $H(C/\tau)$ on C was normal and the intercept corresponded to the formula molecular weight. In the absence of salt, at concentrations above 20 g./l., the points fell on a straight line that seemed to extrapolate to $1/2$ the formula weight. Below 20 g./l., the $H(C/\tau)$ values curved strongly downward toward the formula weight. This behavior is interpreted in terms of ordering effects in the solution, due to electrostatic repulsion.

Introduction

In addition to the use of the light scattering technique for the determination of molecular weights,^{3,4} it has been of special value in the study of physico-chemical properties of proteins⁵⁻⁷ and in the elucidation of processes in which they are involved, such as enzyme inactivation,⁸ enzyme-substrate interaction^{9,10} and aggregation-disaggregation phenomena.¹¹⁻¹⁷ The latter types of studies, however, are complicated by the fact that often the best experimental conditions for the investigation of the given process may be poor from the standpoint of light scattering work, with the result that the data cannot be interpreted in an unambiguous manner.

For a system composed of water, protein and a salt, for example, multi-component theory¹⁸⁻²⁰ yields the following expression for the variation of the excess turbidity, τ , with concentration

$$H \frac{C_2}{\tau} = \frac{1}{1+D} \left\{ \frac{1}{M_2} + \left[\frac{A_{22}}{M_2} - \frac{A_{12}}{M_1} \left(\frac{A_{12}}{\frac{2}{C_1} + A_{11}} \right) \right] C_2 \right\}$$

$$H = \frac{32\pi^3 n^2 (\partial n / \partial C_2)^2}{3\lambda^4 N}$$

$$D = \frac{-2\alpha A_{12}}{\frac{2}{C_1} + A_{11}} + \left(\frac{\alpha A_{12}}{\frac{2}{C_1} + A_{11}} \right)^2 \quad (1)$$

$$A_{ij} = \frac{1}{RT} \frac{\partial \mu_i^{(e)}}{\partial C_j}$$

$$\mu_i = RT \log C_i + \mu_i^{(e)} + \mu_i^0(T, p)$$

$$\alpha = \frac{\partial n}{\partial C_1} / \frac{\partial n}{\partial C_2}$$

where water is component 0, a 1-1 salt is component 1 and protein is component 2. C_i is the concentration of component i in grams per ml., n is the refractive index of the solution, M_i is the molecular weight of component i , and $\mu_i^{(e)}$ is the excess chemical potential of that component. It is apparent from this equation that in systems involving strong salt-protein interaction the zero concentration intercept does not give the molecular weight of the protein. Attraction between protein and salt leads to values of $(HC_2/\tau)_{C_2=0}$ lower than those corresponding to the molecular weight, while repulsion results in values which are too high.

In cases where salt-protein interactions are sufficiently small and can be neglected, equation 1 can be transformed⁷ to

$$H \frac{C_2}{\tau} = \frac{1}{M_2} + \frac{1000}{M_2^2} C_2 \left(\frac{Z_2^2}{2m_1} + \beta_{22} \right) \quad (2)$$

where m is the molarity of the salt and β_{22} is $\partial \ln \alpha_2 / \partial C_2$, i.e., the derivative of the activity coefficient of the protein with respect to its own concentration. Here, extrapolation to infinite dilution yields the correct molecular weight within the above approximation. In many cases, however, the extrapolation cannot be performed, and it is necessary to calculate the molecular weight *directly* from the light scattering data obtained at finite concentrations. Under such conditions serious difficulties may arise.

For moderate protein concentrations, under conditions of low charge and high ionic strength, the value of $H(C_2/\tau)$ is a reasonable approximation of the reciprocal of the weight average molecular weight. At low ionic strength and high charge this approximation can lead to very serious errors. The use of salt-free systems, in which the light scattering expression is formally simpler, does not eliminate these difficulties. For a two-component system, the light scattering equation is

$$H \frac{C_2}{\tau} = \frac{1}{M_2} \left[1 + \frac{C_2}{RT} \left(\frac{\partial \mu_2^{(e)}}{\partial C_2} \right)_{T,p} \right] \quad (3)$$

(1) This work was presented in part at the Second Delaware Valley Regional Meeting of the Am. Chem. Soc., February, 1958, and at the 133rd Meeting of the American Chemical Society, San Francisco, April, 1958.

(2) Eastern Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

(3) M. Halwer, G. C. Nutting and B. A. Brice, *J. Am. Chem. Soc.*, **73**, 2786 (1951).

(4) M. J. Kronman and M. D. Stern, *THIS JOURNAL*, **59**, 969 (1955).

(5) S. N. Timasheff and I. Tinoco, *Arch. Biochem. Biophys.*, **66**, 427 (1957).

(6) S. N. Timasheff, H. M. Dintzis, J. G. Kirkwood and B. D. Coleman, *J. Am. Chem. Soc.*, **79**, 782 (1957).

(7) J. T. Edsall, H. Edelhoch, R. Lontie and P. R. Morrison, *ibid.*, **72**, 4641 (1950).

(8) H. Edelhoch, *ibid.*, **79**, 6100 (1957).

(9) D. S. Yasnoff and H. B. Bull, *J. Biol. Chem.*, **200**, 619 (1953).

(10) M. J. Kronman and M. D. Stern, manuscript in preparation.

(11) J. E. Fitzgerald, N. S. Schneider and D. F. Waugh, *J. Am. Chem. Soc.*, **79**, 601 (1957).

(12) J. F. Foster and R. C. Rhees, *Arch. Biochem. Biophys.*, **40**, 437 (1952).

(13) S. N. Timasheff and R. J. Gibbs, *ibid.*, **70**, 547 (1957).

(14) R. Townend and S. N. Timasheff, *J. Am. Chem. Soc.*, **79**, 3613 (1957).

(15) M. J. Kronman, M. D. Stern and S. N. Timasheff, *THIS JOURNAL*, **60**, 829 (1956).

(16) I. Tinoco, *Arch. Biochem. Biophys.*, **68**, 367 (1957).

(17) M. Bier and F. F. Nord, *Proc. Natl. Acad. Sci., U. S.*, **35**, 17 (1949).

(18) J. G. Kirkwood and R. J. Goldberg, *J. Chem. Phys.*, **18**, 54 (1950).

(19) W. H. Stockmayer, *ibid.*, **18**, 58 (1950).

(20) H. C. Brinkman and J. J. Hermans, *ibid.*, **17**, 574 (1949).

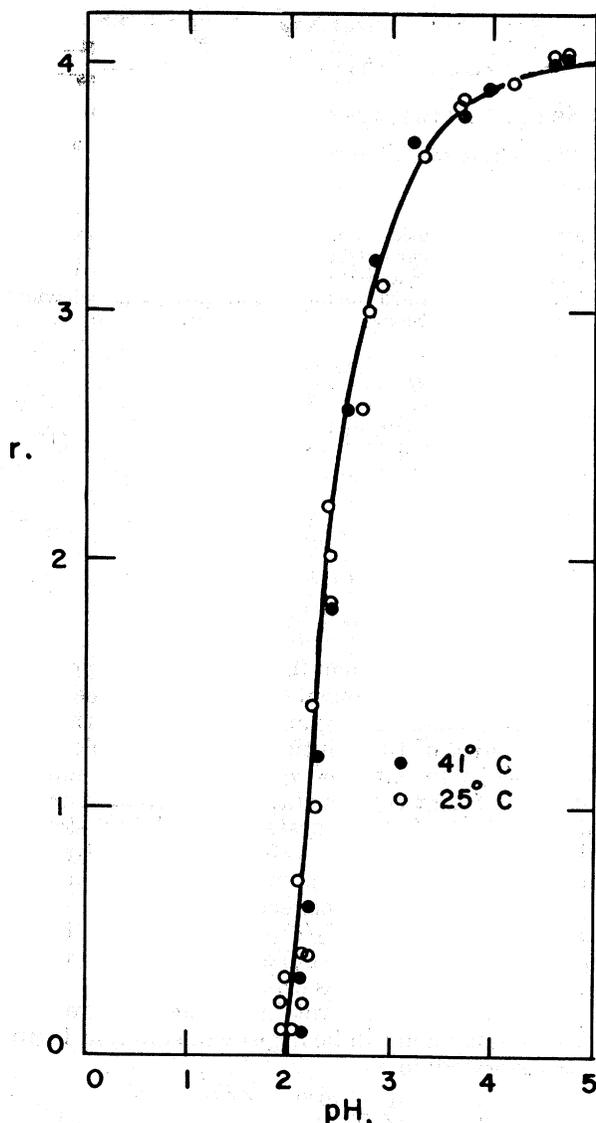


Fig. 1.—The titration curve of STA in the presence of 0.300 *M* NaCl: —, curve calculated assuming STA to be a strong acid. *r* represents moles of base added per mole of STA.

In this case extrapolation of $H(C_2/\tau)$ to infinite dilution does yield $1/M_2$. This extrapolation, however, may be difficult or practically impossible due to the strong and often complicated concentration dependence of $H(C_2/\tau)$. Such is the case found for isoionic proteins^{5,6,21} and for highly charged macromolecules.^{13,22,23}

It is this problem of the interpretation of light scattering data from very low ionic strength solutions of highly charged particles that is considered in this paper. This problem has been examined previously by Doty and Steiner²² for the case of serum albumin at low *pH*'s. The study of such a protein involves certain innate difficulties, since it cannot be considered unequivocally from the viewpoint of a definite physical model, because its shape

(21) J. G. Kirkwood and S. N. Timasheff, *Arch. Biochem. Biophys.*, **65**, 50 (1956).

(22) P. Doty and R. F. Steiner, *J. Chem. Phys.*, **20**, 85 (1952).

(23) A. Oth and P. Doty, *This Journal*, **56**, 43 (1952).

is not truly spherical and its electric charge and charge distribution at any set of conditions may be somewhat uncertain.

The work to be reported in this communication was carried out on silicotungstic acid (STA) which is known to be a spherical molecule,²⁴ 5.5 Å. in radius. Its charge of -4 corresponds to a charge density comparable to that of proteins distant from their isoelectric point. Because of these properties it seemed like a good model system for studying the effect of high charge on the light scattering behavior of macromolecules.

Experimental

Material.—Reagent grade, General Chemical silicotungstic acid (STA) was dried to constant weight over calcium chloride and the residual moisture determined by the Karl Fischer method. Concentrated stock solutions were prepared for light scattering measurements by dissolving the appropriate amount of STA in doubly distilled water and titrating to *pH* 4.5 to 5 with the calculated amount of sodium hydroxide solution.

Light Scattering Measurements.—Measurements of excess turbidity at 436 *mμ* were made with the Brice photometer²⁵ equipped with the narrow slit system. Measurements were made by the Dintzis procedure^{6,26} paying particular attention to the exclusion of dust. Stock solutions were clarified using an ultrafine filter of the type designed by Bier.^{27,28}

The absence of fluorescence was demonstrated by the method described by Brice, Nutting and Halwer.²⁹ Depolarizations were determined in the usual way and found to be negligibly small.^{29,30} Refractive index increments were determined with a photoelectric differential refractometer.³¹

***pH* Measurements.**—The titration curve of STA was determined using a Beckman GS³² differential *pH* meter together with a thermostated electrode assembly similar to that described by Tanford.³³ Standards for these measurements were freshly prepared HCl solutions having the same salt concentration as the solutions with which they were being compared, as well as a *pH* within one unit of that of the solution. Other *pH* measurements were made with a Beckman G *pH* meter.

Electrophoresis Experiments.—Electrophoretic measurements were carried out with a Model 38-A Perkin-Elmer apparatus at 0°. Mobilities were calculated from enlarged projected tracings.

Results

Silicotungstic Acid Charge.—One of the most important parameters in this study being the average net charge of the macro-ion, it was important to determine the *pH* dependence, if any, of the ionization of silicotungstic acid.

Shown in Fig. 1 are results of titration experiments carried out at constant final STA and NaCl concentrations of 0.00197 and 0.300 *M*, respectively.

(24) M. C. Baker, P. A. Lyons and S. J. Singer, *J. Am. Chem. Soc.*, **77**, 2011 (1955).

(25) B. A. Brice, M. Halwer and R. Speiser, *J. Opt. Soc. Amer.*, **40**, 768 (1950).

(26) H. M. Dintzis, in preparation.

(27) M. Bier, Doctoral Dissertation, Fordham University, 1950.

(28) F. F. Nord, M. Bier and S. N. Timasheff, *J. Am. Chem. Soc.*, **73**, 289 (1951).

(29) B. A. Brice, G. C. Nutting and M. Halwer, *ibid.*, **75**, 824 (1953).

(30) E. P. Geiduschek, *J. Polymer Sci.*, **13**, 408 (1954).

(31) B. A. Brice, to be published.

(32) The mention of commercial products throughout this paper does not imply that they are endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.

(33) C. Tanford in T. Shedlovsky, ed., "Electrochemistry in Biology and Medicine," John Wiley and Sons, Inc., New York, N. Y., 1955.

The experimental points obtained at 25 and 40° are shown by the circles, while the solid line was calculated on the assumption that the acid is completely dissociated over the entire pH range. It is thus evident that STA is a strong acid having four ionizable hydrogens, and is completely dissociated over the pH range studied.

A further determination of the variation of charge with pH was obtained from the pH dependence of the electrophoretic mobility. These experiments were carried out at an STA concentration of $1.1 \pm 0.09 \times 10^{-2}$ g./ml. and yielded an average mobility of $-1.56 \pm 0.1 \times 10^{-4}$ over the pH range of 1.71 to 4.64. Making use of the Henry equation³⁴ and properly taking into account the size of the gegenions,³⁵ it was found that this corresponds to a net charge of -3.4 ± 0.3 with no pH variation characteristic of ionization. If one allows for the fact that the experiments were carried out at a finite STA concentration and for the limitations of the Henry equation, the charge of -3.4 obtained from electrophoresis can be considered in satisfactory agreement with the value of -4.0 obtained from the titration curve.

High Ionic Strength Experiments.—Light scattering data on STA obtained in the presence of 0.300 M NaCl are shown in Fig. 2. $H(C_2/\tau)$ was found to be linear in STA concentration with a slope, $2B$, of 1.22×10^{-2} , corresponding to a reduced slope $BM^2/1000$ of 51.3. The latter value is comparable to those reported by Edsall, *et al.*,⁷ for serum albumin at low charge and high ionic strength.

The molecular weight obtained from the intercept of the curve was found to be 2860. In Table I it is compared with values obtained by other methods. The agreement is found to be excellent, especially in view of the errors inherent in the molecular weight determination of relatively small molecules by the light scattering technique.

As is shown in eq. 1 for a three component system, where strong interactions are absent, A_{12} tends to zero. In such a case D is also zero and the intercept yields the correct molecular weight. The fact that the light scattering intercept corresponds to the formula molecular weight of STA, therefore, is evidence that salt-STA interactions are not significant.

TABLE I
MOLECULAR WEIGHT OF SILICOTUNGSTIC ACID

$M \times 10^{-3}$	Method	Ref.
2.86	Light scattering	This study
2.875	Formula weight	
2.91	Sedimentation-diffusion	Baker, <i>et al.</i> ²⁴
2.8	Sedimentation-Archibald procedure	Kronman and Timasheff Unpublished results

The positive slope is indicative of repulsive interactions between quadruply charged silicotungstate ions. Its sign and magnitude are as would be expected from the screening provided by an ionic strength of 0.300.

Salt-free Solutions.—Light scattering data ob-

(34) D. C. Henry, *Proc. Roy. Soc. (London)*, **A133**, 106 (1931).

(35) M. H. Gorin, *J. Chem. Phys.*, **7**, 405 (1939).

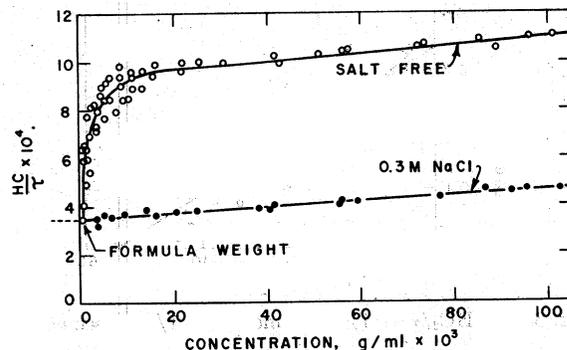


Fig. 2.— $H(C_2/\tau)$ as a function of STA concentration in the presence and absence of neutral salt.

tained in the absence of salt are shown also in Fig. 2. In the region above a concentration of 20 g./l. the data appear to fall on a straight line that would extrapolate to an intercept corresponding to about $1/3$ of the formula molecular weight. At concentrations below 20 g./l. the scattering shows a marked dependence upon concentration, $H(C_2/\tau)$ decreasing almost threefold in the concentration range of 1 to 10 g./l.³⁶

Examination of equation 3 indicates that for a constant M_2 the observed variation of $H(C_2/\tau)$ must be due to either a concentration dependence of $\partial\mu_2^{(e)}/\partial C_2$ or of H . The only portion of H that need be considered is the refractive index increment $\partial n/\partial C_2$. The latter quantity was determined as a function of concentration over the range of 8.57 to 82.3 g./l. The value obtained for 436 $m\mu$ was 0.1065 ± 0.0014 with no significant concentration variation. The refractive index increment for STA in the presence of 0.300 M NaCl was the same as for the salt-free acid within experimental error. Therefore, the variation of $H(C_2/\tau)$ with concentration in terms of equation 3 leads to the conclusion that $\partial\mu_2^{(e)}/\partial C_2$ is a strong function of the STA concentration and that the rapid increase of $H(C_2/\tau)$ with concentration is the result of strong repulsive forces between the silicotungstate ions.

Discussion

Quantitative Representation of the Data.—The large positive values of $\partial\mu_2^{(e)}/\partial C_2$ observed for STA in the absence of salt testify to the presence of strong repulsive forces between the STA particles. This is not surprising since STA is a tetravalent ion and in the absence of salt the screening is extremely weak. The gradual leveling off of the light scattering curve with an increase in STA concentration can be attributed to the growth of screening as the STA concentration increases since the Debye-Hückel factor κ increases directly with the square root of STA concentration. Taking the univalent gegenion concentration into account

$$\kappa^2 = \frac{4\pi N e^2}{DkT} \left(\frac{20C_2}{M_2} \right) \quad (4)$$

where e is the protonic charge, D is the dielectric constant of the medium, k is Boltzmann's constant,

(36) The high scatter of experimental points at low concentrations is not surprising, since in that range the excess turbidity represents only a few per cent. of the apparent turbidity of the pure solvent.

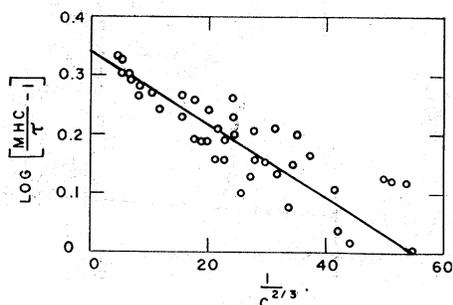


Fig. 3.— $(M_2HC_2/\tau - 1)$ as a function of $1/C_2^{2/3}$ (see eq. 6).

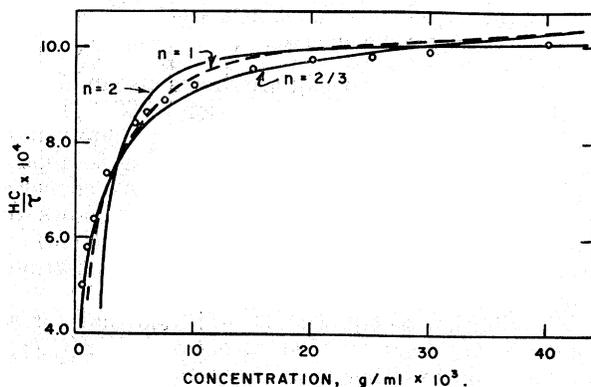


Fig. 4.—Comparison of the smoothed experimental data (circles) with semi-empirical curves obtained from $1/C_2^{2/3}$, $1/C_2$ and $1/C_2^2$ fits.

T is the thermodynamic temperature, C_2 is the STA concentration in g./l. and M_2 is its molecular weight.

A strong repulsive force of the magnitude existing in this system should result in an ordering effect and thus a non-random arrangement of the scattering centers.^{37,38} Qualitatively, one would expect this to result in a diminution of scattered intensity as compared to that from an isolated particle. A quantitative treatment of the problem has been made by Doty and Steiner²² in their study of serum albumin at high charge and very low ionic strength.

Making use of the Fournet radial distribution function³⁹ together with suitable expressions for the repulsive ionic potential, they have derived light scattering equations which describe the observed marked concentration dependence of the scattering for serum albumin under the above stated conditions. For a "soft" exponential potential they obtain the expression

$$\left[\frac{M_2HC_2}{\tau} - 1 \right] = \frac{\pi^{3/2}N_0C_2r_0^3}{M_2} \exp(-h^2r_0^2/4) \quad (5)$$

where $h = 4\pi\lambda' \sin \theta/2$, r_0 is the distance of closest approach of the scattering centers, N_0 is Avogadro's number, λ' is the wave length of the light in the medium and θ is the angle of scattering. It is quite apparent that the greater the screening against electrostatic repulsion the smaller will be r_0 . Thus, in a salt-free solution, r_0 will be some function of the gegenion and hence the macro-ion con-

centration. If it is assumed, as Doty and Steiner have done, that the distance is proportional to $1/C_2^{1/3}$, equation 5 in logarithmic form becomes

$$\log \left[\frac{M_2HC_2}{\tau} - 1 \right] = \log \left(\frac{\pi^{3/2}N_0k^3}{M_2} \right) - \left(\frac{hk}{2C_2^{1/3}} \right)^2 \log e \quad (6)$$

where k is the proportionality constant between r_0 and $1/C_2^{1/3}$.

A plot of the data in the form of equation 6 is shown in Fig. 3. While the plot of $\log ((M_2HC_2/\tau) - 1)$ versus $1/C_2^{2/3}$ appears to be a reasonable fit, plots made versus $1/C_2$ and $1/C_2^{1/3}$ were equally good. The $1/C_2^2$ fit was noticeably poorer. A comparison of the smoothed experimental data with curves calculated using the empirical constants of the $1/C_2^{2/3}$, $1/C_2$ and $1/C_2^2$ fits is given in Fig. 4. While the curves for the exponent of C_2 equal to $2/3$ and 1 seem to describe the data reasonably well at moderately high concentrations, the data fall below the curve at higher values of C_2 . Nonetheless, the form of the curve suggested by equation 5 appears to be adequate.

While it seems possible to predict the shape of the light scattering curve using equation 5, it is by no means as certain that, at the present time, the curve can be calculated theoretically from molecular data. Involved in such a calculation would be an exact evaluation of the relationship between r_0 and macro-ion concentration.

In relating r_0 and C_2 , Doty and Steiner²² made use of equation 2 for a three component system neglecting salt-macro-ion interaction, and assuming β_{22} to be negligibly small. In such a case, a comparison of the coefficients of C_2 in equation 5 and the Donnan term of equation 2 resulted in the previously cited cube root relationship between C_2 and r_0 . Leaving out the β_{22} term in equation 2 is equivalent to assuming that the Donnan term makes the more important contribution to the slope and that the system is close to being ideal. Examination of literature data^{6,7} for serum albumin indicates that this need not be so. Indeed, for most of the conditions cited, $Z_2^2/2m_1$, and β_{22} were of comparable magnitude.

Furthermore, as Doty and Steiner have pointed out,²² equation 2 is inapplicable to a salt-free system. For such a case, multicomponent light scattering theory yields equation 3 which contains no Donnan term and only a β_{22} contribution. No simple relationship can be written, however, between β_{22} and C_2 . Thus, at the present time the problem of the rigorous elimination of r_0 in terms of C_2 from equations, such as eq. 5, must be regarded as unsolved.

A further difficulty, which may or may not be related to the problem discussed above, can be seen when the factor k is calculated using eq. 6 and the experimental data. Such a calculation results in two significantly different values of k : the one obtained from the slope is 8.81×10^{-7} , while the intercept yields 1.23×10^{-7} . This difference is well outside the experimental error. At the present time, it is impossible to state whether this discrepancy is due to the difficulty of relating r_0 and C_2 or to the question of the applicability of the potential functions used to obtain eq. 5 and similar

(37) J. G. Kirkwood and J. Mazur, *J. Polymer Sci.*, **9**, 519 (1952).

(38) J. G. Kirkwood and J. Mazur, *Compt. rend. 8^e Reunion Chim. Phys. Paris*, 143 (1952).

(39) G. Fournet, *Compt. rend.*, **228**, 1421 (1949).

expressions.⁴⁰ In all probability both factors made a significant contribution.

Doty and Steiner²² have found further that in systems displaying a non-random distribution of scattering centers, external interference should result in negative dissymmetries in light scattering. Calculations, however, have shown that in the case of STA, the last would be significant only in the very low concentration range, at which the excess turbidity is very small. Attempts at such measurements have met with failure so far, as the experimental error was found to be greater than the expected difference in scattering at angles of 45 and 135°.

Light Scattering from Other Heteropoly Acids.—

Kerker, Lee and Chou⁴² have reported light scattering data for the 9 and 12 phosphotungstic acids which seem to exhibit the same extreme dependence of $H(C_2/\tau)$ on C_2 . In the absence of salt, the apparent molecular weight of the 12 acid was found to be 35% lower than the formula weight, while for the 9 acid it was 25% lower than the monomer weight. In the case of the 12 acid, the failure of $H(C_2/\tau)$ to decrease sharply with C_2 or even to deviate markedly from linearity is probably

(40) It is interesting to point out that calculation of k from a light scattering expression involving the Verwey-Overbeek potential⁴¹ also results in a discrepancy. In this case, however, the intercept yields a value of k much larger than that obtained from the slope.

(41) E. J. W. Verwey and J. T. G. Overbeek, "Theory of the Stability of Lyophobic Colloids," Elsevier Publishing Co., Inc., Amsterdam, 1948.

(42) M. Kerker, D. Lee and A. Chou, *J. Am. Chem. Soc.*, **80**, 1539 (1958).

due to the fact that the measurements were not carried to concentrations sufficiently low to detect the strong curvature. As Fig. 2 indicates, at a concentration of 20 g./l., which is comparable to the lowest concentration employed by Kerker, Lee and Chou, $H(C_2/\tau)$ for STA is still linear in C_2 .

In the case of the 9 acid, the slight curvature that Kerker, Lee and Chou observed at low values of C_2 is consistent with our results and the expectations from the theory.²² Quantitative comparisons of our results with those obtained for the phosphotungstic acids is impossible since the latter measurements do not extend to sufficiently low concentrations for the use of expressions such as eq. 5.

Conclusion

The above-described light scattering measurements on silicotungstic acid can be considered as good experimental evidence for the non-random arrangement in solutions of macro-ions under conditions of low screening. Although no rigorous quantitative treatment of the experimental data is available at the present time, the theory of Doty and Steiner can be used as a first approach for the analysis of such systems. It yields good agreement with experimental data within the limits of the assumptions in its derivation. A more exact theoretical analysis of the situation would seem desirable, and work to that effect has been initiated.

Acknowledgment.—The authors wish to thank Professor S. J. Singer for suggesting this problem and Dr. B. A. Brice for his encouragement in the course of these studies.