(1.6% in chloroform). For the absorption spectrum, see Fig. 7 and Table I.

Vitamin D_3.—Vitamin D_3 3,5-dinitrobenzoate (5.78 g., 0.01 mole) was refluxed with 22 ml. of 10% methanolic potassium hydroxide (0.04 mole, 2.2 g.) for ten minutes under an atmosphere of nitrogen. The reaction mixture was stirred to insure homogeneity. The saponification being completed, the deep purple mixture was cooled quickly to room temperature and poured into 35 ml. of water and extracted twice with 30 ml. of hexane. The combined hexane extracts were washed with distilled water until free of alkali and dried over anhydrous sodium sulfate. The solvent was removed in a vacuum, leaving a stiff resinous residue. The resinous vitamin was dissolved in 7 g. of acetone and cooled in an ice–salt-bath. Water was added to the cold solution until cloudiness developed, and the solution was then allowed to stand at 2° for three days, during which time the vitamin slowly crystallized out. After this time water was again added until the solution was cloudy, after which it was allowed to stand for three days at 2°. The crystalline vitamin D_3 was filtered and washed repeatedly with small portions of cold 50% aqueous acetone and then dried in vacuum over calcium chloride; yield, 1.9–3.0 g. (50–80%), m. p. 84–85°, $[\alpha]_D$ +51.9° (1.6% in chloroform). For the absorption spectrum, see Fig. 7 and Table I.

Provitamins and Vitamin Esters.—One one-hundredth of a mole of the crystalline alcohol was dissolved in 0.18 mole of pyridine (C. P., freshly distilled and dried over barium oxide) and the mixture treated with 0.012 mole of the freshly prepared acid chloride. The mixture was agitated until no further heat was liberated and then allowed to stand at room temperature for fifteen hours. The reaction mixture (an orange to red solution over precipitated pyridine hydrochloride) was poured into excess water and exhaustively extracted with benzene. The benzene layer was then extracted, twice each, successively, with 10% aqueous oxalic acid and cold 10% sodium carbonate, and washed with water until neutral. The solution was then dried over sodium sulfate and the solvent removed by vacuum distillation. The solid residue was recrystallized until there was no further change in melting point, rotation and ultraviolet absorption.

Vitamin D_3 3-nitro-4-methylbenzoate prepared by this method was a resin which could not be crystallized.

The vitamin D_2 4-nitrobenzoate was quite difficult to purify.

Cyclohexyl Esters.—These esters were prepared from C. P. cyclohexanol by the method described above.

Physical data, excluding ultraviolet absorption, for all the esters are given in Table IV.[27]

Acknowledgments.—The authors wish to express their appreciation to Mr. J. R. McCormick for frequent assistance, to Miss E. Rossi for technical work, and to the Misses E. Bass, P. Curran and A. Rainey for the microanalyses.

Summary

1. The ultraviolet absorption spectra of several nitrobenzoic esters of the provitamins and vitamins D_2 and D_3 have been determined and compared with the spectra of the corresponding free sterols and acids and also the cyclohexyl esters of the same acids. The comparisons were made by a graphical subtraction method.

2. Several previously unreported esters have been prepared.

[27] The ultraviolet absorption data for all the materials studied are available in the form of tables and graphs (with the exception of those presented in the text) as a supplement to this paper, in a document of the American Documentation Institute, 1719 N Street, Washington 6, D. C.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL]

Studies on Double Refraction of Flow. II. The Molecular Dimensions of Zein

BY JOSEPH F. FOSTER AND JOHN T. EDSALL

The production of birefringence in certain normally isotropic liquids through the action of shearing stress has long been known. Not until about 1916, however, was the phenomenon associated with the orientation of asymmetric particles. Recent theoretical and experimental advances, of which two comprehensive reviews have recently been published,[1,2] have nevertheless clearly demonstrated the potentialities of this method in the elucidation of molecular size and shape.

In the case of the very elongated protein molecules such as myosin and tobacco mosaic virus, orientation can be brought about by low gradients (of the order of 100–500 sec.$^{-1}$), and a relatively simple apparatus suffices. Snellman and Björnståhl,[3] and Sadron, *et al.*,[4] through the use of instruments providing much higher gradients, have been able to extend the method to less elongated protein molecules, such as helix hemocyanin and horse antipneumococcus serum globulin, obtaining results which agree very satisfactorily with sedimentation and diffusion data.

The recent development[5] in this Laboratory of an instrument providing velocity gradients as high as 30,000 sec.$^{-1}$, together with the use of solvents of high viscosity, has made possible the extension of the method to some corpuscular proteins of even lower molecular weight. The present paper presents some results obtained with zein, the prolamine of corn.

Theoretical

The phenomenon of flow birefringence is best observed when the liquid is placed between two concentric cylinders, one of which is rotated while the other is held fixed. If the field between the cylinders is observed between crossed Nicol prisms, with light traveling parallel to the cylinder

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[1] J. T. Edsall, in "Advances in Colloid Science," Interscience Publishers, New York, N. Y., 1942, Vol. 1, p. 269.

[2] J. T. Edsall, in E. J. Cohn and J. T. Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943, p. 506.

[3] O. Snellman and Y. Björnståhl, *Kolloid-Beihefte*, **52**, 403 (1941).

[4] C. Sadron, *J. phys. radium*, [7] **7**, 263 (1936); C. Sadron, A. Bonot and H. Mosimann, *J. chim. phys.*, **36**, 78 (1939).

[5] J. T. Edsall, C. G. Gordon, J. W. Mehl, H. Scheinberg and D. W. Mann, *Rev. Sci. Instruments*, **15**, 243 (1944).

axes, the flowing liquid appears optically like a uniaxial spherocrystal. The field appears bright, except for four dark regions spaced like the arms of a cross (the "cross of isocline"). In these regions the optic axis of the solution is parallel to the plane of vibration of the light transmitted by either the polarizer or the analyzer. It is found experimentally that at very low velocity gradients the arms of the cross bisect these planes at 45°. As the gradient is increased, the position of the cross shifts, and at very high gradients approaches coincidence with the planes of vibration. The smaller of the two angles between these planes and an adjoining arm of the dark cross is known as the extinction angle, χ. Thus, χ tends from 45° at very low to 0° at very high gradients. At values near 45°, χ is a nearly linear function of the velocity gradient G, and the increment $-\mathrm{d}\chi/\mathrm{d}G$ increases very rapidly with increase in the major axis of the asymmetric particle being studied. At higher gradients, the χ-G curve of course becomes flatter, but the gradient required to achieve a given χ value is always much smaller for a long particle than for a short one.

It may readily be deduced from the above definition,[1,2,3] that χ is the angle between the stream lines and the optic axis in the flowing liquid—this axis being so defined as to lie always between 45 and 0° relative to the stream lines. The refractive index for light vibrating with its electric vector parallel to this axis is denoted by n_e; for light vibrating perpendicular to it, by n_0. The sign and magnitude of the double refraction is given by the value of $n_e - n_o$. In general, $n_e - n_o$ is a nearly linear function of G when χ is near 45° and approaches a limiting saturation value as χ approaches 0°.

These phenomena can be interpreted in terms of molecular orientation by the velocity gradient in the flowing liquid, opposed by the rotary Brownian movement which produces disorientation and a tendency toward a purely random distribution. The intensity of this Brownian movement may be characterized by a rotary diffusion constant, Θ. The fundamental treatment of this problem, for very thin rod-shaped particles, was given by Boeder[6]; the treatment has been generalized, and extended to rigid ellipsoids of revolution[7] of any axial ratio, by Peterlin and Stuart[8,9] and by Snellman and Björnståhl.[3]

The statistical distribution of the orientations of the molecules can be described as a function of the parameter $\alpha = G/\Theta$, and of the axial ratio (a/b) of the molecules.[10] At very low velocity gradients, the degree of orientation is small, and the most favored orientation is at 45° to the stream lines. As the velocity gradient increases, the orientation becomes more pronounced, and the angle of most favored orientation moves over toward the stream lines. It is this prevailing orientation relative to the stream lines which determines the extinction angle, χ. The fraction of all the molecules present, the axes of which are oriented at or near this most favored direction, determines the magnitude of the double refraction.

The theoretical calculation of χ as a function of α leads to a general equation defining $\tan 2\chi$ as a function of the orientation distribution function of the molecules. At low α values ($\alpha < 2$) this assumes the simple form

$$\tan 2\chi = 6/\alpha = 6\Theta/G \qquad (1)$$

or expressing χ in radians, and including one higher term

$$\frac{\pi}{4} - \chi = \frac{\alpha}{12}\left[1 - \frac{\alpha^2}{108}\left(1 + \frac{24a^2}{35b^2}\right) + \cdots\right] \qquad (2)$$

The double refraction in the flowing solution is given by the equation

$$n_e - n_o = \frac{2\pi\Phi}{n_s}(g_1 - g_2)\cdot \mathrm{f}(\alpha, a/b) = \frac{2\pi\Phi}{n_s}(g_1 - g_2)\frac{\alpha a}{15b} \times \left[1 - \frac{\alpha^2}{72}\left(1 + \frac{6a^2}{35b^2}\right) + \cdots\right] \qquad (3)$$

Here Φ is the volume fraction of the solute molecules, n_s the refractive index of the solution at rest, $g_1 - g_2$ an optical factor which depends only on the geometry of the solute molecule and the refractive indices of solvent and solute, and $\mathrm{f}(\alpha, a/b)$ is the orientation factor.

For α values below 2, corresponding to χ values between 45° and about 36°, the term in brackets in equation (1) can be ignored, and α is a linear function of the observed χ value.[11] Since $\alpha = G/\Theta$, and G is directly determined from the dimensions of the apparatus and the speed of the rotating cylinder, the value of Θ is thereby fixed.

It now remains to relate Θ to the size and shape of the molecule. This relation has been calculated for prolate and oblate ellipsoids of revolution.[12,13] For prolate ellipsoids, with which we shall be chiefly concerned here, the rotary diffusion constant is quite accurately given by the equation

$$\Theta = \frac{1}{2\tau} = \frac{3kT}{16\pi\eta a^3}\left(2 \ln \frac{2a}{b} - 1\right) \qquad (4)$$

provided the axial ratio (a/b) is greater than 5.

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(6) P. Boeder, *Z. Physik*, **76**, 258 (1932).

(7) We shall not attempt here to consider the behavior of molecules, of the type represented by many high polymers, which may be regarded as long flexible chains, capable of deformation by the stresses produced by the velocity gradient in the flowing liquid. Such systems have recently been considered in great detail by W. Kuhn and H. Kuhn, *Helv. Chim. Acta*, **26**, 1394 (1943). Evidence from various sources, especially ultracentrifugal and dielectric dispersion measurements, indicates that most proteins, including zein, are relatively rigid structures of fairly wide cross sections (see, for example, ref. 2, especially Chapter 22, by J. L. Oncley).

(8) A. Peterlin, *Z. Physik*, **111**, 232 (1938).

(9) A. Peterlin and H. A. Stuart, *ibid.*, **112**, 1, 129 (1939).

(10) Here a is the semi-axis of revolution, b the equatorial semi-axis. If $a > b$, the ellipsoid is prolate, of length $2a$; if $a < b$, it is oblate (disk-shaped) of diameter $2b$.

(11) This presupposes that the concentration of solute molecules is so low that χ, for a given value of the velocity gradient, does not vary with concentration. If this is not the case, the molecules are so close together that they do not orient independently.

(12) R. Gans, *Ann. Physik*, **86**, 628 (1928).

(13) F. Perrin, *J. phys. radium*, [7] **5**, 497 (1934).

The term in the logarithm of a/b varies relatively little, even for fairly wide variations in a/b itself. Hence a rough estimate of the axial ratio, derived for instance from viscosity measurements, is adequate to fix this factor with sufficient accuracy. Knowing the temperature (T) and the viscosity of the solvent (η) it is then possible to calculate the length ($2a$) of the molecule from the Θ value determined by equation (1) or (2). The simple relation given in (4), between the rotary diffusion constant Θ and the relaxation time τ, should be noted.[1,2,12,13,14] It indicates that direct comparisons are possible between relaxation times determined from dielectric dispersion measurements and those determined from double refraction of flow. Such a comparison in the case of zein is given later in this paper.

Reference to equation (4) will show that an equation of the form

$$\eta\Theta/T = f(a, a/b) \qquad (5)$$

can be written. Thus, $\eta\Theta/T$ should be independent of variations in the temperature, or the viscosity of the solvent employed, provided these variations do not alter the size and shape of the molecule. This provides a most enlightening method of comparing results in different media, at different temperatures and gradients. However, in the case of a polydisperse solute one measures a weighted average value of $\eta\Theta/T$. Sadron[15] has shown mathematically how the various components weight the resulting value of χ and Δ. Qualitatively the result is just as one would expect intuitively; at low gradients the more asymmetric molecules play a preponderant role, but, as the orienting force increases, the shorter and less asymmetric molecules become effective. Hence, in a polydisperse system one would expect that the calculated value of $\eta\Theta/T$ would start at a value corresponding to the component of lowest rotary diffusion constant and increase with increasing gradient. Thus a graph of $\eta\Theta/T$ *vs.* $G\eta$ gives some insight into the degree of homogeneity of a solute system.

The measurements of double refraction are less susceptible than the χ values to interpretation in terms of molecular size and shape. They do, however, furnish valuable indications as to the optical characteristics of the molecule, which can throw light on its molecular configuration. Moreover, some changes in protein molecules, such as those produced by certain denaturing agents, are reflected with great sensitiveness by marked changes in the value of $n_e - n_o$.

Experimental Methods

Concentric Cylinder Apparatus.—A detailed description of the apparatus is given elsewhere.[5] In brief, it consists of two concentric cylinders mounted in a vertical position with a light source and polarizing Nicol prism mounted above the cylinders, and an analyzing Nicol and viewing telescope mounted below them. The radius of the inner cylinder is 2.51 cm. and the gap between the cylinders is 0.25 mm. wide. The velocity gradient, G, is given by the equation

$$G = \frac{R\omega}{d} = \frac{2\pi RN}{60d} \qquad (6)$$

Here ω is the angular velocity of the rotor—in this case the inner cylinder—N is the speed of the rotor in r. p. m., R the mean radius of the cylinders and d the gap width. For the present apparatus G, expressed in sec.$^{-1}$, is equal to 10.5 times the speed in r. p. m.

The inner cylinder is driven by a one-sixth H. P. direct current motor, wired in such a manner that the potential across the field and armature circuits can be varied independently, thus providing steady speeds over a wide range. A belt drive with variable pulley ratios further extends this range (*ca.* 75–3000 r. p. m.). The outer cylinder is cooled by circulating water and the speed of rotation determined stroboscopically. The cylinders are constructed of stainless steel of a type found to be inert to protein solutions.

The light source, a type H-3 General Electric high pressure mercury arc, is focused by a condenser on a slit placed at the focal point of a second lens. The light emerging from the second lens is nearly plane parallel; it then traverses in succession the polarizer, the gap between the cylinders, and the analyzer, finally entering the observing telescope. The polarizer and analyzer are mounted so that they can be rotated independently for $n_e - n_o$ measurements or connected by a bar and rotated simultaneously in the crossed position for measurement of the extinction angle. The light can be passed through the solution between the cylinders by means of two small optical glass windows placed above and below the gap on one side. The visible field thus constitutes only a few degrees of arc and only one arm of the cross of isocline is observable at a time.

In measuring the extinction angle, χ, the crossed Nicols are rotated until one arm of the cross coincides with a cross hair in the telescope and the reading obtained from a vernier scale, on the analyzer mount, which is graduated to 0.05 degree. The average of several settings is always used, at least three readings being made even under the most favorable conditions. The arrangement of the motor and drive shaft permits rotation of the cylinders with equal ease in both senses. Measurements of χ are always determined for rotation in both senses at any given speed and the value taken is the mean of the two values so obtained. The method of calibrating the reference plane of the Nicol prism has been given elsewhere.[5,16]

Measurement of the magnitude of the double refraction is made by means of the Sénarmont compensator previously used by von Muralt and Edsall[16] and others. It consists simply of a quarter wave plate with its optic axis parallel (or perpendicular) to the planes of transmission of the polarizer which in turn is placed at 45° to the optic axis of the solution. The initially plane polarized light upon passing through the birefringent solution is converted, in general, to elliptically polarized light. This is converted, in turn, by the properly oriented $\lambda/4$ plate, to plane polarized light which has been rotated, however, through an angle Δ with respect to the original plane of polarization. The magnitude of Δ is related to the magnitude of double refraction by the equation

$$\Delta = \frac{180S}{\lambda_o}(n_e - n_o) \qquad (7)$$

where S is the path length of the light in the birefringent medium and λ_o the wave length of the light *in vacuo*. Substituting the value of S for the apparatus used here, namely, 7 cm., and the wave length of the mercury green line, 5461 Å., this relation becomes

$$n_e - n_o = 4.24 \times 10^{-8}\Delta \qquad (8)$$

Δ being expressed in degrees.

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[14] J. L. Oncley, THIS JOURNAL, **60**, 1115 (1938).

[15] C Sadron, *J. phys. radium*, [7] **9**, 381 (1938).

[16] A. L. von Muralt and J. T. Edsall, *J. Biol. Chem.*, **89**, 315, 351 (1930).

In making the measurements of Δ the analyzer is first set at 45° to the position of the cross of isocline. Then with the cylinders at rest, using a half-shadow wedge to obtain greater precision, the polarizer is crossed with the analyzer and a quarter wave plate introduced and set at the extinction position. Several readings are now made of the extinction setting of the analyzer. The cylinders are then set in rotation and several more readings made. The difference between the means of the two series of readings is obviously equal to Δ.

Whereas monochromatic light should theoretically be used it was found that essentially the same results are obtained with or without filters. Furthermore, it was found possible to make $n_e - n_o$ measurements over a range of gradients without resetting the Nicols and quarter wave plate, so long as χ did not vary more than about five degrees.

Errors Arising from Reflections.—One of the most serious difficulties encountered in the use of the present apparatus arises from the effect of light rays reflected from the cylinder walls. Frey-Wyssling and Weber[17] have considered the effect of reflections, pointing out that the resulting depolarization has a maximum and minimum, upon rotating the Nicols, similar to a flowing solution for which $\chi = 0°$. The superposition of this effect on the double refraction of the flowing solution may give false values of both χ and Δ. The error is greatest at low gradients where the double refraction of the flowing solution is least. Frey-Wyssling and Weber[17] have suggested a vectorial method of correcting for this effect by determining $n_e - n_o$ as a function of angle for the solution at rest. Their method is, however, cumbersome and depends on the assumption that the magnitude of the reflections is the same in the flowing solution as in the solution at rest. This may be far from true, especially if thermal gradients are set up in the solution.[18]

The alternative course of attempting to minimize the effect has been chosen. Björnståhl[19] has suggested methods for eliminating reflections, none of which has been found adaptable in the present case due to the extreme narrowness of the gap (see ref. 5). However, by careful alignment of the optical system, it has been found possible to obtain values of χ in which considerable confidence can be placed so long as Δ (equation 7) is not too small.

For measurements at low Δ values, the relative intensity of the reflected light can be much reduced by greatly increasing the length of the path traversed by the light between the cylinders and the observer. This was achieved by passing the light down through a hole in the table, into a blackened box, which extended to the floor, about three feet below. The light was reflected back from a mirror, placed at the bottom of the box and inclined at a very small angle to the horizontal, and observed at table level with the observing telescope pointing downward. A lens, covered except for a slit about 2 mm. wide, was mounted at table level in such a manner that it could be shifted laterally to select the center of the beam ensuing from the cylinders. Only rays traveling very nearly parallel to the axis of the cylinders can traverse this long light path and be received within the relatively small aperture of the lens and the observing telescope. This technique does not completely eliminate the reflected light, but it does greatly reduce its intensity relative to that of the light transmitted through the liquid without reflection. Measurements by this method, though more accurate, are considerably less precise than those obtained by focusing directly on the cylinders, using a short light path. Hence, when the long optical path was used, five to ten readings were taken at each gradient, in each sense of rotation. Average values and probable errors were calculated. The errors were usually of the order of 0.5 to 1.3°, as contrasted to a maximum of 0.5° (provided Δ exceeds *ca.* 10°) by the direct method. In general, this method was used only at low gradients and low protein concentrations, where the errors due to reflections are greatest.

The accuracy of the observed reading was determined by preliminary experiments employing ethyl cinnamate, which gives a fairly high double refraction. As for all pure liquids composed of small molecules, $\chi = 45°$ at all attainable gradients[20] for ethyl cinnamate. Thus the deviations from 45° for the observed values give an index of the error to which these readings are subject (Table I). It is obvious that the readings at the lowest recorded gradient are quite unreliable, but the others are reasonably satisfactory, and readings at Δ values above 15° should be subject to little error.

Similar trial runs with ethyl cinnamate were made at occasional intervals, between the runs on protein solutions, to ensure that no significant disturbance in the alignment of the apparatus had occurred in the meantime.

TABLE I

OBSERVED VALUES OF EXTINCTION ANGLE FOR ETHYL CINNAMATE[a]

G (sec.$^{-1}$)	χ Direct observation	χ (Long optical path)	Δ°
3,800	52.6 ± 1.81	48.9 ± 1.57	4.6
9,500	46.4 ± 0.52	45.0 ± 0.66	10.3
15,000	44.5 ± .32	43.9 ± 0.68	17.4

[a] Theoretically χ should be 45° at all gradients.

Zein Preparations.—Four zein preparations were investigated in the present work.

(1) **Standard Zein** was a commercial sample[21] prepared from corn gluten at elevated temperature. It contained considerable pigment and a small amount of material which flocculated spontaneously out of aqueous–alcoholic solution upon standing.

(2) **Washed Standard Zein** was prepared from Standard Zein by washing thirteen times by suspending in distilled water, allowing to settle and decanting. This was designed to remove inorganic impurities and any other water soluble material. The product gave solutions much less colored than those of the unwashed material, thus making optical measurements more reliable.

(3) **Fraction II-1** was prepared by alcohol–water precipitation of Standard Zein at 0°. Details of the fractionation will not be given here. Suffice it to say that this fraction was of intermediate solubility and probably representative of the bulk of Standard Zein. It was relatively free of gross impurities and pigment.

(4) **Laboratory Zein** was prepared in this Laboratory from white corn. The entire process, including grinding of the corn, was carried out at 0° or below. The zein was extracted from the corn with 72% (by vol.) isopropyl alcohol and the solution was clarified by passage through a pulp filter before precipitation in ice water. The product gave beautifully clear, colorless solutions which were unusually stable.

Viscosity Increments of Zein Preparations.—The viscosity increments[22] of the various zein preparations were

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[17] A. Frey-Wyssling and E. Weber, *Helv. Chim. Acta*, **24**, 278 (1941). These authors also discuss the effect of anisotropy in the windows of the apparatus. This effect is, however, nil in the present apparatus.

[18] See Y. Björnståhl, *Z. Physik*, **119**, 245 (1942).

[19] Y. Björnståhl, *J. Optical Soc. Am.*, **29**, 201 (1939).

[20] Molecules of this size possess rotary diffusion constants of the order 10^{10} sec.$^{-1}$. This means that gradients of the order 10^9 sec.$^{-1}$ must be attained before χ falls measurably below 45°.

[21] Supplied through the courtesy of the Corn Products Refining Company, Argo, Illinois.

[22] The viscosity increment is defined as the limiting value at infinite dilution of the function $(\eta/\eta_0 - 1)/\Phi$ where Φ is the volume fraction of solute (see for example ref. 2, p. 515). In computing axial ratios from viscosity increments the equations of Simha (*J. Phys. Chem.*, **44**, 25 (1944)) were used (summarized in ref. 2, p. 519), effects of solvation being ignored. It is of interest that M. A. Lauffer (THIS JOURNAL, **66**, 1191 (1944)) has found Simha's equation to give excellent agreement with results obtained by other physical measurements in the case of tobacco mosaic virus, a protein having an axial ratio of the same order of magnitude as zein in spite of its enormously greater molecular weight.

TABLE II

SUMMARY OF RESULTS

Preparation	Solvent	T, °C.	Concn., g./100 ml.	Viscosity, in poises, of solvent, η	Range of $G\eta \times 10^{-3}$	$(\Delta/G\eta C)_0 \times 10^9$	$\Theta \times 10^{-2}$	$\eta\Theta/T$	Length in Å. (if prolate)
Standard	Propylene glycol	21–22	2.79	0.47–0.51	0.5 –4.4	7.5	8.20	13.6	315
	Propylene glycol	20–21	2.79	.51– .54	.75–5.5	...	9.50	13.5	315
	Propylene glycol	24	1.89	.41	1.10–4.30	6.3	11.6	16.3	295
Washed standard	Propylene glycol	19.5–20.5	2.91	.51– .55	1.08–5.01	8.0	8.20	15.3	305
	Propylene glycol	19.5–20.7	2.22	.50– .55	1.80–5.60	6.9	8.35	15.3	305
	Propylene glycol	21.4–22.1	0.97	.47– .50	1.89–5.92	6.7	9.30	15.3	305
	Propylene glycol	4.6–5.0	.97	1.50–1.58	2.58–11.5	6.3	3.90	20.5	275
Fraction II-1	Propylene glycol	21–24	3.0	0.43–0.51	0.58–3.90	...	5.90	9.5	360
	Propylene glycol	21.4–22.4	1.95	.47–0.50	.67–5.92	7.2	7.95	13.1	335
	Propylene glycol	5.7–6.1	1.95	1.43–1.47	1.46–10.8	8.5	3.10	16.3	295
	Propylene glycol	21.0–21.6	1.00	0.49–0.51	1.55–5.30	6.3	8.20	13.9	330
	Propylene glycol	4.0–4.3	1.03	1.62–1.66	5.10–12.5	...	3.55	21.0	270
	75–25 (by vol.) P.G.–EtOH	20.2–20.6	1.92	0.17	0.54–2.65	8.0	13.0	7.51	385
	50–50 P.G.–EtOH	20.2–20.6	1.92	.069	.43–1.28	8.9	18.0	4.25	460
	73%–EtOH–H_2O	19.2	1.93	.023	.43[a]	8.3	70[b]	5.4[b]	425[b]
	73%–EtOH–H_2O	4.6	1.93	.037	.35– .70	7.9	160[b]	20[b]	275[b]
Laboratory	Propylene glycol	20.5–21.1	2.94	.51–0.53	1.06–4.81	9.0	3.08	5.45	425
	Propylene glycol	17.0–22.3	1.91	.47– .67	0.58–5.02	9.5	3.85	7.50	385
	Propylene glycol	20.3–21.2	0.99	.50– .54	2.04–6.30	7.7	4.50	8.00	375
	Propylene glycol	4.8–5.0	.99	1.55	6.10–15.0	...	1.97[c]	10.4[c]	345[c]

[a] Measurement made at one gradient only. [b] Little if any significance can be attached to these values. The experimental error is very high. [c] Only the measurement at the lowest gradient was used for purposes of calculation. Beyond this the isocline was too far below 45° for the linear relation to apply.

determined in 73% ethanol with and without added salt. Plots of $(\ln \eta_R)/C$ *vs.* $\ln \eta_R$ were linear and could be readily extrapolated. The viscosity increments of Standard Zein and the two preparations derived from it were identical within the experimental error, 25.0 ± 0.5. The fact that Fraction II-1 has the same increments as the crude material is of interest since in the fractionation process materials of much lower and higher increments (ranging from 22 to 30) were obtained. Fraction II-1 is probably representative of the bulk of Standard Zein but is undoubtedly more homogeneous.

Laboratory Zein, on the other hand, had a significantly higher increment, 32.0 ± 0.5. This corresponds to an axial ratio of 18:1 calculated for a prolate ellipsoid from Simha's equations or to 1:46, calculated for an oblate ellipsoid. The corresponding axial ratios for the other three preparations are all identical, being 15:1 (prolate) or 1:35 (oblate).

Viscosity measurements were also made on Fraction II-1 in propylene glycol. The increment in this medium was identical with that in aqueous alcohol within the experimental error. It thus appears that no appreciable difference in molecular shape is involved in this change of solvent, which is, of course, essential to any comparison of results in the two media such as will be made later.

Solvent.—Propylene glycol was used as solvent in most of the runs. Not only is it an excellent solvent for zein (even better than ethanol–water as evidenced by the lower cloud-point and higher stability to gelation of propylene glycol solutions) but its high viscosity greatly increases the degree of orientation attainable.[23] Eastman Kodak Co. material (density, 1.0324 at 25°) was used without further purification or dehydration. The viscosity of the solvent was determined in a capillary over the temperature range 0–30°. Over this temperature range the viscosity could be expressed conveniently and accurately by the linear equation

$$\log \eta = 2.34 - 0.0307t \qquad (9)$$

where η is in centipoises and t is the temperature in degrees centigrade.

Preparation of Solutions.—The zein was weighed out and the proper amount of solvent added by weight. As soon as solution was complete (in most cases this required about an hour) the solution was evacuated to remove dissolved air (this step is important in eliminating formation of bubbles in the apparatus) and studied immediately. In a few cases, not reported here, the solutions were allowed to stand for long periods of time. No significant changes in behavior resulted from standing in propylene glycol solution, even up to two months.

Some studies were made of the effect of protein charge by adding acid and alkali to the propylene glycol solutions. The effects were negligible in all cases, except when the maximum acid or base binding capacity was approached. All of the results reported in the present paper were on zein solutions of unadjusted *p*H and hence represent the behavior of the isoelectric protein.

Discussion of Results

The results of a representative series of runs on the four zein preparations are presented in Table II. In most cases there was some variation in temperature during the runs so that the extremes are given together with the over-all variation in solvent viscosity. The values of $G\eta$ given are the limits as calculated from the highest and lowest gradients together with the viscosities corresponding to the temperature appropriate to each gra-

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(23) This effect of high solvent viscosity is two-fold. First, and most important, it resists disorientation of the particles so that at a given gradient the degree of orientation is approximately proportional to the viscosity. In addition, however, it has been shown by Taylor, *Phil. Trans. Roy. Soc. (London)*, **A223**, 289 (1923), that for the case of flow of a liquid between concentric cylinders, the inner of which is rotating, there exists a critical velocity above which flow will no longer be laminar, but turbulent. This critical velocity is approximately proportional to the viscosity of the liquid. Thus, whereas with the present instrument turbulence would set in at gradients of about 6600 sec.$^{-1}$ with water at 20°, in the case of propylene glycol the critical velocity is far above the attainable limit.

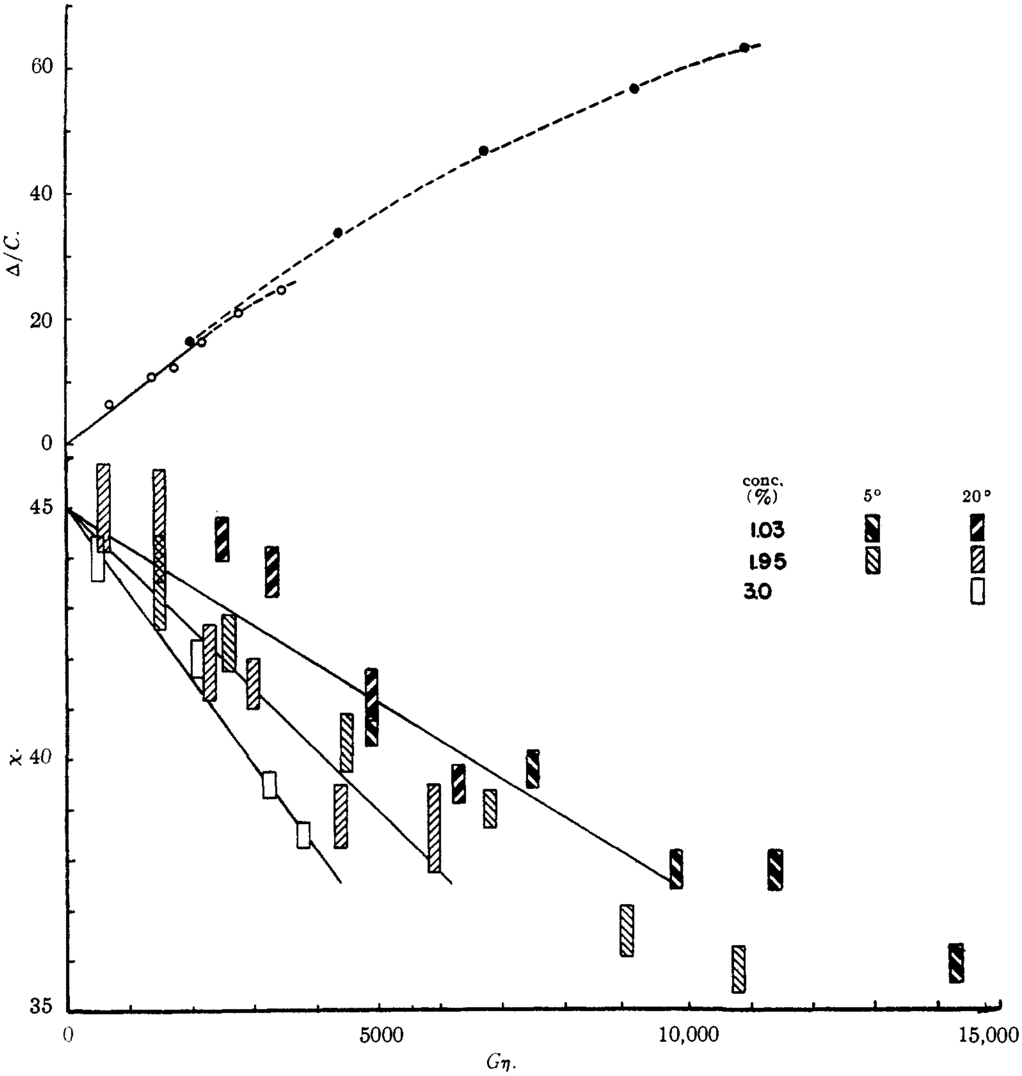

Fig. 1.—Dependence of Δ/C (upper curve) and χ (lower curve) on $G\eta$ for Fraction II-1 in propylene glycol. In the upper curve the open circles correspond to measurements at room temperature, the full circles to low temperature (5°), all for 1.95% protein solution. The probable errors in the extinction angle measurements are indicated by the vertical height of the rectangles.

dient. In general, readings were made at from five to eight gradients.

The other quantities in Table II are calculated as follows. A plot of Δ/C (the observed rotation of the Sénarmont compensator divided by the protein concentration in g. per 100 ml.) *vs.* $G\eta$ was made (as in Fig. 1). This was usually linear up to $G\eta$ values of about 2000, and the slope of this linear portion is given as $(\Delta/G\eta C)_0$ in column 7. Similarly χ was plotted against $G\eta$ and the best straight line drawn to fit the data down to χ values of about 38–40°. In this region the term in α^2 in equation (2) is negligible and the simple linear relation

$$\chi = \pi/4 - \alpha/12 \qquad (10)$$

where χ is in radians, holds. It follows that $\alpha = 1.00$ when $\chi = 40.3°$. Hence, from the definition of α, $\eta\Theta$ is equal to the value of $G\eta$ at which $\chi = 40.3°$. This quantity divided by T, the absolute temperature, is given in column 9 of Table II. The value of Θ given in column 8 is obtained from $\eta\Theta$ by dividing by the mean viscosity and hence is not strictly so reliable as $\eta\Theta/T$.

The value of the length, given in column 10, is computed with the aid of equation (4), assuming the molecule to be a prolate ellipsoid of axial ratio 20:1. The assumed axial ratio is a rounded value based on the axial ratios calculated from viscosity, and is accurate enough for the purpose (the resulting length would be altered only about 5% by assuming an axial ratio of 15 or 35).

Dependence of χ and Δ on $G\eta$.—In Fig. 1 the results of some measurements on Fraction II-1 in propylene glycol are given in the form of plots of the extinction angle (χ) and double refraction (expressed as Δ/C) plotted against the product of velocity gradient and solvent viscosity, $G\eta$. These results are typical of those obtained with the other preparations.

The shape of these curves is what would be expected from theoretical considerations and from previous results on other materials. There is an initial linear region in both the χ and Δ/C curves, deviations from linearity setting in at gradients corresponding to χ values of 38–40°.

It is obvious, from inspection of Fig. 1, that the probable errors in χ become large at low gradients and low protein concentrations. In addition to this error of measurement, however, is the previously discussed error due to reflections, which probably accounts for the tendency of the observed values at low gradients to lie above the mean curves as drawn (see the data on calibration in Table I). This effect is of course also greatest at low protein concentration and is, in fact, apparently completely negligible in the curve for 3% protein.

Concentration Dependence.—If the zein molecules are sufficiently far apart to orient independently, χ and Δ/C at a given $G\eta$ value should not vary with the protein concentration. The experimental curves reveal a small but definite dependence on concentration which indicates some interaction. Thus, for example, in Fig. 1 there is a downward trend in χ with increasing protein concentration. In the case of Laboratory Zein as seen in Table II, Δ/C is essentially the same in the 2.94 and 1.91% solutions, but falls off somewhat in the 0.99% solution; while $\eta\Theta/T$ shows a definite rise with decreasing protein concentration. These effects would be expected qualitatively if there is appreciable interaction between protein molecules. All in all the effects are not large, especially when expressed in terms of calculated molecular length (for example, the difference between the room temperature results at the lowest and highest concentration in Fig. 1 corresponds to only about 10% in length). It would be desirable to extend the studies to lower protein concentration but the resulting double refraction would be so low that the results would have little meaning. Even in 1% solutions the probable error is high. Nevertheless it seems safe to conclude that interaction plays only a minor role in these solutions and that the results give reasonably accurate measures of the rotary diffusion constant.

Dependence of $\eta\Theta/T$ on $G\eta$.—In Fig. 2 $\eta\Theta/T$ is plotted as a function of $G\eta$ for three of the zein preparations, using the data obtained at 3% concentration in each case. In the case of Washed Standard Zein and Fraction II-1, $\eta\Theta/T$ is constant within the experimental error, while with Laboratory Zein there is some suggestion of an increase which is outside the experimental error. This is not an isolated case, but is characteristic of all the runs on the laboratory preparation, and in view of the preceding discussion on the properties of the function $\eta\Theta/T$, might indicate more heterogeneity as to length in this material. However, it does not seem safe to draw such a conclusion until the theory is more fully substantiated. For this purpose a material of suitable molecular size and shape and known homogeneity is almost essential.

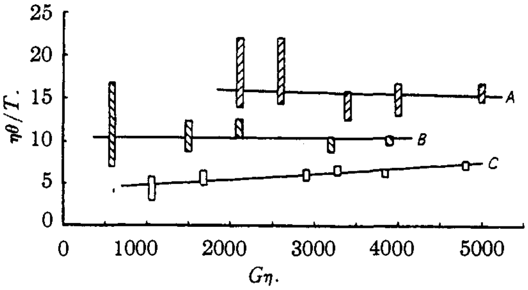

Fig. 2.—Dependence of the function $\eta\theta/T$ on $G\eta$. The vertical heights of the rectangles indicate probable errors. The three curves correspond to Washed Standard Zein (A), Fraction II-1 (B) and Laboratory Zein (C). All measurements at room temperature.

Temperature Dependence.—Figure 1 indicates no very marked discontinuity between the isocline curves obtained at room and low temperatures. However at $G\eta$ values of 2000 to 5000 where the curves at the two temperatures overlap, the χ values at low temperature appear to lie appreciably higher. This is reflected in the calculated $\eta\Theta/T$ and length values, as is seen in Table II. This cannot be explained by aggregation of zein at low temperatures, since this would produce a shift in the opposite direction to that observed.

Mechanical heating of the solution may well account for part or all of this effect in which case the room temperature results would give a closer approximation to the truth.[24]

Solvent Dependence.—In Fig. 3 a study is made of the effect of varying the solvent. Zein II-1 was studied in several propylene glycol–ethanol mixtures. There seems to be a definite trend in the observed χ values, those in pure propylene glycol being the highest. Change in molecular shape upon adding alcohol appears unlikely since, as shown by the viscosity measurements previously mentioned, no marked change in molecular shape is involved even in going from 73% aqueous alcohol to propylene glycol. The

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(24) Unfortunately only the outer of the two cylinders is water cooled. In the room temperature runs the inner cylinder is possibly a little above the temperature of the circulating water, but certainly not much. At the low temperature, however, the inner cylinder may be considerably above the temperature of the water jacket, in which case there would be a temperature gradient. The recorded water jacket temperature would, of course, be the minimum. This effect also complicates the reflection problem. Björnståhl[18] has treated the effect of gradients of refractive index, produced by thermal gradients arising from the heat generated in the flowing solution. Such gradients can certainly arise, and may lead to reflections of light from the cylinder walls, even when the light entering the liquid is perfectly plane parallel. Björnståhl's very detailed theoretical treatment, however, assumes that the inner and outer cylinders are at the same temperature, an assumption which certainly does not correspond to the conditions actually existing in our apparatus.

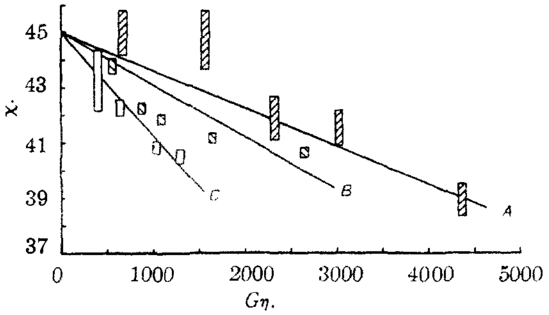

Fig. 3.—Extinction angle curves for Fraction II-1 as a function of the solvent composition. Curve A, pure propylene glycol; curve B, 75:25 vol. ratio of propylene glycol to ethanol; curve C, 50:50 vol. ratio. All measurements at room temperature. The vertical heights of the rectangles indicate probable errors.

most likely explanation seems to lie in greater interaction between the solute molecules as the proportion of alcohol is increased. This explanation would be in qualitative agreement with an observation made that addition of alcohol to propylene glycol solutions of zein raised the cloud point, *i. e.*, diminished the effectiveness of the solvent.

Comparison with Results of Sedimentation and Diffusion Studies.—Williams and Watson[25] have reported sedimentation and diffusion results on three zein fractions, the main component having the values $s_{20,w} = 1.9S$ and $D_{20,w} = 4.0 \times 10^{-7}$ sq. cm./sec. These constants, together with the value 0.77 for the partial specific volume,[26] correspond to a molecular weight of 50,000 and an f/f_0 ratio of 2.17. If the form of the zein molecule is assumed to be that of a prolate ellipsoid this would correspond to an axial ratio of 25:1 and a length of 425 Å.

In view of the heterogeneity of zein preparations it is difficult to make any quantitative comparisons between various physical methods. Preliminary measurements[27] of the sedimentation constants indicate that Laboratory Zein is comparable to the main component of Williams and Watson ($s = 1.9$) while the other three preparations used in the present study have lower sedimentation constants (*ca.* 1.6). The lengths given in Table II for Laboratory Zein agree very well with the value 425 Å. computed in the preceding paragraph. The lower sedimentation constants of the other preparations, together with their lower axial ratios, can be interpreted as indicating shorter molecules. The results in Table II for these preparations are in qualitative agreement with this conclusion.

Comparison with Dielectric Dispersion Results.—The only physical measurements on zein with which the present results do not agree at least reasonably well, appear to be the dielectric dispersion measurements of Elliott and Williams.[28] These workers found values of the relaxation times, for rotation about the major and minor axes, of 4.2 and 24 × 10^{-8} sec., respectively, in water at 25°. On this basis (assuming prolate ellipsoids) they computed a molecular weight of 38,000 and axial ratio of only 7:1. The present results yield relaxation times, when reduced to water at 25°, of the order of 100 to 325 × 10^{-8} sec. for the various fractions. These values should, of course, be compared with the greater of the two relaxation times observed by dielectric dispersion, but even so there is a discrepancy of 4 to 10 fold.[29]

Calculation for Oblate Ellipsoids.—For oblate ellipsoids of high axial ratio Θ becomes independent of the small axis and inversely proportional to the cube of the major axis.[1,2,14] Thus for highly asymmetric oblate ellipsoids a molecular diameter can be calculated without regard to molecular thickness. Using the value 8.0 for $\eta\Theta/T$, as observed for Laboratory Zein at the lowest concentration, the calculated diameter is 240 Å. For a molecular weight of 50,000, as estimated for this zein preparation, the molecular thickness would have to be of the order 2 Å. Of course this is absurd for a protein molecule. Furthermore this would give an axial ratio a/b of 1:120 which does not agree at all with the value estimated for oblate ellipsoids from viscosity (1:46) and from sedimentation and diffusion (1:40). A combination of flow double refraction results with the other physical data available, thus strongly favors a prolate type model for the zein molecule.

Sign of the Double Refraction.—The sign of the double refraction was in all cases positive with respect to the direction of orientation.

Acknowledgment.—The authors are indebted to Prof. E. J. Cohn, Dr. J. L. Oncley and Dr. Herbert Scheinberg for their interest, suggestions and assistance; to Dr. D. French for coöperating in the Zein Fractionation; and to Drs. C. C. Jensen and C. R. Harmison who made the Laboratory Zein preparation.

Summary

The theory of double refraction of flow and its application to the determination of molecular length is reviewed briefly. Through the use of a new apparatus providing velocity gradients as high as 30,000 sec.$^{-1}$ together with the technique of high viscosity solvents (propylene glycol) it has been possible to study by this method a relatively

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[25] J. W. Williams and C. C. Watson, "Cold Spring Harbor Symposia Quant. Biol.," **6**, 208 (1938).

[26] J. F. Foster and D. French, THIS JOURNAL, **67**, 687 (1945).

[27] These studies are being made by Dr. J. L. Oncley of this Department.

[28] M. A. Elliott and J. W. Williams, THIS JOURNAL, **61**, 718 (1939).

[29] Unpublished dielectric dispersion measurements by J. L. Oncley in this Laboratory on Washed Standard Zein give curves which require the assumption of at least three different relaxation times for complete fitting of the experimental data. An estimate of the longest of these indicates a value $\tau^{25°}_{H_2O}$ of 50–100 × 10^{-8} sec., in fair agreement with the present results.

short, corpuscular protein, zein. The results are in very satisfactory agreement with sedimentation, diffusion and viscosity measurements on this protein when compared on the basis of a prolate ellipsoidal molecule roughly 300–400 Å. in length.

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[CONTRIBUTION FROM THE DEPARTMENT OF FUEL TECHNOLOGY OF THE PENNSYLVANIA STATE COLLEGE]

The Chemistry of Nitrogen in Humic Acids from Nitric Acid Treated Coal[1]

BY H. B. CHARMBURY, J. W. ECKERD, J. S. LATORRE AND C. R. KINNEY

The partial oxidation of coals yields acidic substances which resemble the naturally occurring humic acids of peat and brown coals in many respects. A particularly effective oxidizing reagent for this purpose is concentrated nitric acid because of the rapidity[2] with which it attacks coal and yet does not carry the oxidation appreciably beyond the humic acid stage. Similar results have been obtained with 1 N nitric acid,[3] but a longer period of heating is required. Nitrogen, as well as oxygen, is introduced into the coal and while the character of the oxygen-containing groups has been fairly well established[2,3,4] nothing appears to be known about the nitrogen groups. In an attempt to find a method for determining the nature of these groups, a study has been made of the action of alkaline permanganate under a variety of conditions on these humic acid-like compounds as well as on selected nitrogen-containing substances and the original coal for comparison.

The coal from which the humic acids were made was of the high volatile A bituminous rank and was taken from the Upper Freeport bed in central Pennsylvania. An analysis appears in Table II. The humic acids were prepared by heating the coal with concentrated nitric acid followed by extraction with alkali and precipitation with dilute hydrochloric acid. Four variations in the method of treating the acids were used. Procedure 1 consisted of determining (a) the amount of ammonia which was liberated from the humic acids by vigorous oxidation with alkaline permanganate, (b) the amount of nitrogen converted to the nitrate ion, and (c) the amount of carbon oxidized to carbon dioxide and volatile acids. Procedure 2 consisted of determining the amount of nitrogen in the humic acids which could be distilled as ammonia by alkaline hydrolysis before oxidizing them with permanganate and continuing as in Procedure 1. Procedure 3 consisted of alkaline hydrolysis, followed by alkaline reduction with zinc, and continuing as in Procedure 1. Procedure 4 consisted of alkaline hydrolysis and reduction in one operation, and then by following Procedure 1.

The results of the experiments on the humic acids and the known compounds are presented in Table I. Those on the humic acids following Procedure 1 indicate that there are at least two forms of nitrogen present. The first of these was liberated as ammonia by alkaline permanganate and amounted to 21.9%. The second was converted to the nitrate ion and amounted to 68.4%. The first form is undoubtedly in a more reduced state because the ammonia was removed in the presence of a strongly oxidizing reagent. The second form appears to be in a higher state of oxidation because it was liberated as the nitrate ion.

Confirmation that a reduced form of nitrogen may be liberated as ammonia by alkaline permanganate oxidation and that an oxidized form of nitrogen may be converted to the nitrate ion was obtained with the known nitrogen compounds appearing in Table I. The amino group in the glycine was freed as ammonia by permanganate oxidation to the extent of 88.5%, while in the case of the more resistant *p*-aminobenzoic acid, only 66.7% of the amino nitrogen was released as ammonia. At the same time 1.0% of the nitrogen in glycine and 8.4% in *p*-aminobenzoic acid was converted to nitrate ion. Under similar conditions only 0.2% of the nitrogen in *p*-nitrobenzoic acid was liberated as ammonia while 39.8% was converted to the nitrate ion. These results indicate that the amount of nitrogen converted to ammonia and nitrate ion by permanganate oxidation depends to a very large degree upon the nature of the nitrogen-containing groups. The low yield of nitrate from *p*-nitrobenzoic acid was undoubtedly due to the resistance of the compound to oxidation because only 21.1% of the carbon was liberated as carbon dioxide and volatile acids. With the humic acids, on the other hand, extensive oxidation occurred and it was possible to account for 93.8% of the carbon and 90.3% of the nitrogen.

Alkaline hydrolysis also liberated ammonia from the humic acids,[5] Procedure 2, Table I, but it was not expected that the quantity would be the same, within the experimental error, as that released by boiling alkaline permanganate. At the same time over half of the remaining nitrogen which appears to be in a higher state of oxidation was reduced to the point where it was evolved as ammonia upon subsequent oxidation with per-

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(1) Presented before the Division of Gas and Fuel Chemistry, American Chemical Society, New York, N. Y., September 14, 1944.

(2) Fuchs and Sandhoff, *Fuel*, **19**, 45, 69 (1940).

(3) Juettner, Smith and Howard, THIS JOURNAL, **57**, 2322 (1935).

(4) Chowdhury and Biswas, *J. Indian Chem. Soc.*, **19**, 289 (1942).

(5) Fuchs and Horn, *Brennstoff-Chem.*, **12**, 60 (1931), first observed this property.