acids HSO_4^-, $HONHSO_3H$ and $HON(SO_3)_2H^-$ are all equal. After lumping several very insensitive terms (terms which vary little with time) into the integration constant, the following integral is obtained for equation (13) for the cases in which no acid was added initially

$$\log \frac{x}{a-x} + C \log (a-x) = \frac{k_B a}{2.303\,(a+K)}\, t + \text{const.} \qquad (14)$$

where K is the acid ionization constant for HSO_4^-, $HON(SO_3)_2H^-$ and $HONHSO_3H$; C is a constant, less than unity in most cases, and affects the linearity of the log $x/a - x$, t curves only slightly. Equation (14) differs little from equation (3), and it is for this reason that straight lines were obtained in Fig. 1.

As a first approximation

$$k_B(a+b)/(a-b+K) = k'(a+b) \qquad (15)$$

and therefore, $k_B = k'(a + K)$ for those runs in which no acid was added initially. In order to obtain values of k_B which may be subjected to inspection, the values of K for HSO_4^- calculated by Pedersen[4] were used, those for the other acids not being known.

The values obtained for k_B are tabulated in the last column of Table II; for zero ionic strength $k_B = 1.5 \times 10^{-2}$ min.$^{-1}$ at 25°, and should be nearly independent of μ. When $\mu < 0.1$, k_B is effectively constant as may be seen in the table.

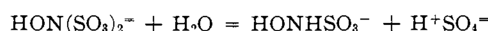
(4) Pedersen, *J. Phys. Chem.*, **38**, 601 (1934).

That the mechanism assumed provides a satisfactory explanation for the experimental results is shown by their accord with the experimentally found rate equation and by the fact that the rate constant k_B varies but little with the ionic strength.

Summary

Anhydrous potassium hydroxylamine disulfonate, $K_2HON(SO_3)_2$, has been prepared and kept more than a month without appreciable decomposition.

The rate of the hydrolysis



$$HON(SO_3)_2^= + H_2O = HONHSO_3^- + H^+SO_4^=$$

has been measured. For acid solutions the rate equation was found to be

$$-d(\Sigma HON(SO_3)_2^=)/dt = k(\Sigma H^+)(\Sigma HON(SO_3)_2^=)$$

where the sigmas refer to the total concentration of all ions containing the group indicated. Water also catalyzes the hydrolysis, but its effect is much less than that of acid. The effect of temperature on k corresponds to an energy of activation of 17,600 cal./mole.

A mechanism which explains the observations satisfactorily assumes that $HON(SO_3)_2H \cdot H_2O^-$ is the intermediate whose rate of decomposition into the final products determines the over-all rate of the hydrolysis.

PASADENA, CALIFORNIA RECEIVED MAY 2, 1941

[CONTRIBUTION FROM THE LABORATORY OF PHYSICAL CHEMISTRY, UNIVERSITY OF WISCONSIN]

The Dielectric Behavior of Solutions of the Protein Gliadin[1]

BY P. P. ENTRIKIN[2]

In the first sedimentation study of gliadin, the prolamine of wheat, Krejci and Svedberg[3] found the protein to be inhomogeneous, a result to be expected in view of the earlier careful and detailed analysis of the material by Haugaard and Johnson.[4] In the sedimentations both whole and fractionated gliadins were used. A sedimentation equilibrium experiment with an approximately homogeneous gliadin fraction which had the same specific sedimentation velocity or sedimentation constant, $s_{20} = 2 \times 10^{-13}$ cm./sec./dyne, as that of the main constituent of purified gliadin gave a value of 27,000 for the molecular weight of the substance. Measurable differences in sedimentation constant of the several gliadin fractions were not observed. Later on the diffusion constants of three gliadin fractions were determined by Lamm and Polson,[5] making it possible to calculate the molecular weight of the predominant constituent. For this fraction the diffusion constant was reported to be $D_{20} = 6.7 \times 10^{-7}$ so that

(1) More complete details of this work are to be found in the thesis of Paul P. Entrikin submitted to the faculty of the University of Wisconsin in partial fulfillment of the requirements for the Ph.D. degree in June, 1940.

(2) Present address: Technical Division, Standard Oil Company of Louisiana, Baton Rouge, Louisiana.

(3) Krejci and Svedberg, THIS JOURNAL, **57**, 946 (1935).

(4) Haugaard and Johnson, *Compt. rend. trav. Lab. Carlsberg*, **18**, No. 2 (1930).

(5) Lamm and Polson, *Biochem. J.*, **30**, 528 (1936).

the molecular weight value to correspond is 27,500. These figures have been retained in the latest tables of Svedberg and Pedersen.[6] Again, Arrhenius,[7] in observations of the shapes of dielectric constant–frequency curves for gliadin solutions has interpreted his results to indicate a molecular weight of 27,000 for the gliadin units.

On the other hand, accurate determinations of the amounts of certain amino acids in gliadin have been used in the estimation of the minimal molecular weight of gliadin to give 42,000[8] and osmotic pressure measurements in concentrated urea and in water solutions give from 40,000 to 44,000[9] for this quantity. Naturally one wonders how to account for these differences. This is especially true when it is remarked that molecular weight data for zein, the alcohol soluble protein from corn, are in good agreement, whether they have been obtained from composition (39,000), from osmotic pressure (38,000), from sedimentation velocity and diffusion experiments (40,000), or from dielectric constant dispersion experiments (38,000).[10] In the desire to aid in the assignment of a more definite molecular weight for gliadin there have been performed a series of dielectric constant dispersion experiments with solutions of fractionated gliadins over a much wider frequency range than has been heretofore attempted.

Theory

The application (and modification) of the Debye–Clausius–Mosotti theory to the anomalous dispersion of dielectric constant of protein solutions and in the computation of the molecular weight of these macromolecules has been given in previous publications from this Laboratory.[11] Molecular weight and time constant are simply related if the solute molecule is spherical, otherwise additional shape factors are required. In the case where the molecule is assumed to have the shape of an elongated ellipsoid of revolution there will be two time constants, τ_1 for a rotation about the short axis and τ_2 for a rotation about the long axis. The necessary shape factors have been given by Perrin.[12]

(6) Svedberg and Pedersen, "The Ultracentrifuge," Clarendon Press, Oxford (1940).

(7) Arrhenius, *J. Chem. Phys.*, **5**, 63 (1937).

(8) Cohn, *Chem. Rev.*, **24**, 203 (1939).

(9) Burk, *J. Biol. Chem.*, **124**, 49 (1938).

(10) Elliott and Williams, THIS JOURNAL, **61**, 718 (1939).

(11) Williams and Oncley, *Physics*, **3**, 314 (1932); Williams, *Trans. Faraday Soc.*, **30**, 723 (1934); Elliott and Williams, THIS JOURNAL, **61**, 718 (1939).

(12) Perrin, *J. phys. radium*, **5**, 497 (1934).

Experimental

The apparatus used in these experiments permitted dielectric constant determinations of solutions of the gliadin fractions to be made over the frequency interval 2.5×10^4 to 3×10^7 cycles per second (λ = 12,000 to 10 meters). The radio-frequency bridge served for the frequencies 2.5×10^4 to 1.5×10^6 cycles per second and the resonance assembly was used for the range 1.5×10^6 to 30×10^6 cycles per second. For statements concerning the form and use of the equipment the reader is referred to the report of Elliott and Williams.[10]

Preparation, Fractionation and Characterization of Gliadin.—A number of different methods were tried in the preparation of the gliadin samples used in these experiments. Procedures which involve the use of ether, absolute alcohol and other organic solvents were avoided because it was feared the physical properties of the protein might in this way be altered. The method of Haugaard and Johnson which involves the use of electrolyte solutions in the separation of the components gave good protein preparations but it was always difficult to electrodialyze the solutions down to the low electrical conductance required for the dielectric constant determinations.

The most satisfactory protein solutions for the dielectric constant studies were obtained by the method recently described by Arrhenius.[7] In a typical preparation 2500 g. of bleached wheat flour was mixed thoroughly with enough 56% aqueous alcohol to cover it readily, and the mixture was agitated for forty-eight hours in a closed vessel. The supernatant liquid was filtered twice through broadcloth and the extraction of the flour was repeated with a second portion of aqueous alcohol. The solution from these two extractions was combined with that obtained by squeezing the residual flour mass in a broadcloth sack. After a period of sedimentation the clear solution above was siphoned off to give what may be described as the whole gliadin in solution. It contained very little starch.

Fractionation of the gliadin has been described by Haugaard and Johnson and our procedure was adjusted to give a comparable separation. The whole gliadin solution was chilled to 0° for forty-eight hours. The clear supernatant liquid was decanted from the precipitate and placed in a brine-bath at −10° again for forty-eight hours. Again a precipitate formed and the clear solution above was separated as before. In this way there were obtained three gliadin fractions, each of which was further purified by alternate solution and precipitation procedures. The protein precipitated at 0° contained the starch impurity, and this substance could be largely removed without difficulty. In the text of this report the three fractions are referred to as gliadins A, B and C. They were all and always completely soluble in 56% aqueous alcohol. For the dielectric constant determinations the solutions were always electrodialyzed.

In order to characterize the three gliadin fractions diffusion constant determinations were carried out. Since similar experiments have already been performed by Lamm and Polson[13] comparisons of the fractions may be made. Our observations were made by using apparatus, solutions, and procedures which are very much like and just as pre-

(13) Lamm and Polson, *Kolloid-Z.*, **87**, 149 (1939); *Biochem. J.*, **30**, 528 (1936).

cise as those of Lamm and Polson. The scale line displacement–distance plots were always very similar to theirs for the corresponding fractions of gliadin. The results of these observations serve to identify our fractions with those used in the Upsala laboratory. Differences in diffusion constant are due more to the difficulties of the fractionation process than to errors inherent in the physical measurement. The method of "standard deviations" was used in the computations for the diffusion constant. The comparison of our data with those of Lamm and Polson is given by the accompanying table.

TABLE I
COMPARISON OF DIFFUSION CONSTANT DATA FOR GLIADIN FRACTIONS

Gliadin fraction (P. P. E.)	$D_{25} \times 10^7$	$D_{20} \times 10^7$	Gliadin fraction (L. and P.)	$D_{20} \times 10^7$
A	5.04	4.46	I	4.96
B	6.36	5.62	II	5.85
C	6.82	6.03	III	6.72

Results

After many preliminary observations, three complete dielectric constant–dispersion experiments were made with the aqueous alcohol solutions of each of the gliadins A, B and C. As representative of these experiments we present in Table II dielectric constant–frequency data for typical solutions of Gliadins A, B and C in 56% aqueous alcohol at 25°. Included with the data in the table are the constants used in obtaining the two theoretical dispersion curves and certain figures to express the dielectric increments and the size and shape of the fractionated gliadin molecular kinetic units. Actual experimental points and theoretical curves (only) to represent these data are brought together to form Fig. 1. As in the case of the zein work the time constants used to describe the two theoretical curves (characteristic of rotations about the long and short axes) have been adjusted to make the composite theoretical curve fit the experimental data as well as possible (see Table II).

TABLE II
DIELECTRIC CONSTANT–FREQUENCY DATA FOR SOLUTIONS OF GLIADIN FRACTIONS, TEMPERATURE 25°

Solvent, 56% aqueous ethyl alcohol, with $\epsilon_{\text{solv.}} = 47.3$; $\eta_{\text{solv.}} = 0.0230$.

Log ν	Gliadin A $C = 13.5$ g./l.	Gliadin B[a] $C = 3.53$ g./l.	Gliadin C $C = 2.87$ g./l.
	Dielectric constant ratio (r)		
	Bridge Apparatus		
4.428	1.1800	1.0358	1.0282
4.545	1.1758	1.0354	1.0274
4.846	1.1498	1.0336	1.0268
5.147	1.1182	1.0298	1.0235
5.448	1.0873	1.0225	1.0179
5.749	1.0606	1.0154	1.0124
5.925	1.0483	1.0127	1.0103
6.050	1.0359	1.0105	1.0084
	Resonance Apparatus		
6.128	1.0305	1.0089	1.0032
6.484	1.0125	1.0027	1.0005
6.739	1.0070	1.0013	1.0007
6.896	1.0065	1.0010	1.0005

[a] These data are plotted to form Fig. 1. The constants used in obtaining theoretical dispersion curves and other pertinent data are here tabulated.

$(r_1)_0 = 1.036$ $(r_2)_0 = 1.014$
$(r_1)_\infty = 1.014$ $(r_2)_\infty = 1.000$
$\log \nu_c = 5.35$ $\log \nu_c = 6.21$
$\tau_1 = 71.1 \times 10^{-8}$ $\tau_2 = 9.81 \times 10^{-8}$
$\tau_1^0 = 27.3 \times 10^{-8}$ $\tau_2^0 = 3.87 \times 10^{-8}$
$\tau_{\text{sph}}^0 = 3.06 \times 10^{-8}$
$\vartheta_1 = 2{,}310$ $\Delta_1 = 0.061$
$\vartheta_2 = 1{,}540$ $\Delta_2 = 0.041$
$\vartheta_{\text{av}} = 3{,}850$ $\Delta_{\text{av}} = 0.102$

$\vartheta = \frac{\Delta\epsilon}{\Delta C}$ where C is in moles/liter, $\Delta = \frac{\Delta\epsilon}{\Delta C}$ where C is in g./100 ml.

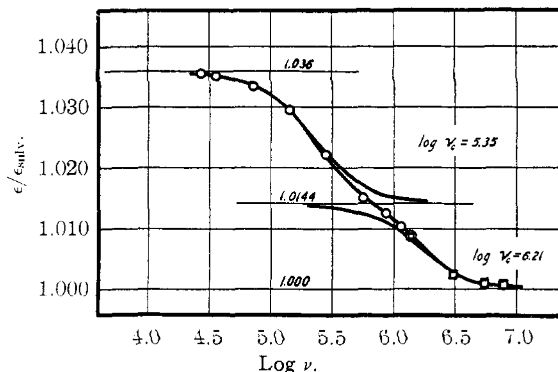


Fig. 1.—Dielectric constant ratio–frequency data for Gliadin B.

The physical constants obtained from the nine complete dielectric constant–frequency experiments are summarized in Table III.

For the equations and an outline of the methods used in the calculations for the several quantities given in this table reference may be made to the earlier article in which similar studies with the zein are reported.[10]

Discussion

The series of dielectric constant–dispersion determinations with fractionated gliadins lead to certain rather interesting conclusions. Inspection of Table III shows that the gliadin fractions B and C do not differ greatly as regards dielectric behavior. In each case the experimental dielectric constant–frequency observations are expressed with a considerable degree of accuracy by the addi-

TABLE III
PHYSICAL CONSTANTS OF GLIADIN FRACTIONS A, B AND C

Property	Gliadin "A"	Gliadin "B"	Gliadin "C"
C (g./l.)	3.6	6.3	5.2
	21.7	3.5	3.5
	13.5	4.4	2.9
$\tau_1^0 \times 10^8$	49.5	28.6	26.6
	48.4	27.3	26.1
	50.8	27.6	25.5
$\tau_2^0 \times 10^8$	5.62	4.03	3.85
	5.20	3.87	3.77
	5.34	3.89	3.61
$\tau_{sph}^0 \times 10^8$	4.36	3.16	3.04
	4.04	3.06	2.98
	4.15	3.14	2.85
M	53,700	39,000	37,400
	49,750	37,700	36,700
	51,200	38,600	35,000
$1/\rho$	9.1	8.0	7.8
	9.8	7.9	7.8
	9.4	7.9	8.0
Δ_1	0.0734	0.0700	0.0563
	.0726	.0613	.0598
	.0810	.0715	.0592
Δ_2	.0493	.0330	.0408
	.0482	.0408	.0370
	.0516	.0403	.0383
Δ_{av}	.1227	.1030	.0971
	.1208	.1021	.0968
	.1326	.1118	.0975
ϑ_{av}	6,575	4,035	4,015
	6,020	3,850	3,555
	6,790	4,310	3,415

tion of two theoretical dispersion curves. From the point of view of this electrical study the two fractions are nearly monodisperse and largely identical. The two relaxation times are those of a single species of non-spherical molecules having two characteristic axes. The middle fraction does contain some heavier molecules and the light fraction has some smaller units present.

The situation is quite different in the case of gliadin A for which the experimental dielectric constant observations cannot be reproduced by the addition of two simple theoretical dispersion curves. There is no difficulty as regards the data at the intermediate frequencies but both the high frequency and low frequency values for the dielectric constant ratio are always too high, indicating a wide spread of molecular size along with elongated molecular shape. Gliadin A makes up about 30% of the whole protein.

In an earlier series of dielectric constant determinations with gliadin fractions Arrhenius made measurements over the frequency interval 2×10^5 to 8.5×10^6 cycles per second (λ = 1500 to 35 meters) and reported $\tau_2^0 = 3.70 \times 10^{-8}$ sec. (rotation about the long axis) and molecular weight of 27,000. The measurements did not extend over a sufficiently large frequency interval to permit an evaluation of the time constant for rotation about the short axis. Since the data were insufficient to give both relaxation times it is to be expected the τ_2^0 value and with it the molecular weight figure will be somewhat too low. Our dielectric constant data indicate very definitely a molecular weight in the neighborhood of 38,000 for the predominant gliadin constituent.

It must be reasonably certain from this and other evidence that the molecular weight for gliadin is higher than 27,000. As was noted a sedimentation equilibrium experiment with a gliadin fraction corresponding nearly to our gliadin C gave a minimum value of M = 26,800, but some drift in the values is apparent. Also, it seems safe to assume sufficient time was not allowed for the establishment of equilibrium, a fact which is sufficient in itself to account for a low result. In the combination of sedimentation velocity and diffusion constant to give the molecular weight the diffusion constant for the lightest and most soluble fraction has been used. It seems to us just as reasonable to use with the sedimentation constant which has always been observed $s_{20} = 2.1 \times 10^{-13}$, the diffusion constant for the middle gliadin fraction, *i. e.*, $D_{20} = 5.85 \times 10^{-7}$. These data give a molecular weight of 34,000 for the predominant gliadin constant. With our D_{20} value, 5.62×10^{-7}, the molecular weight is calculated to be 36,000.

The dielectric constant determinations lead to a shape factor 8 to 1 for the ratio of major to minor axis of the gliadin unit. Lamm and Polson give 10 to 1 as a result of their diffusion studies for this ratio. However, this calculation is based upon the values M = 27,000 and $D = 6.72 \times 10^{-7}$. By using the data of this report M = 36,000 and $D = 5.62 \times 10^{-7}$ the dissymmetry number is 1.7 and the axis ratio is about 13 to 1. These ratio figures are much like the ones which are obtained in the same way for zein. It is of interest that these are practically the only molecules belonging to the 40,000 molecular weight class which have such a high dissymmetry. It is possible that these prolamines may have been unfolded or opened up due to a reaction caused by the use of alcohol in bringing them into solution.

Application of the classical Debye theory and of a modified theory of Onsager would lead to

widely different values for the dipole moment of gliadin. We have preferred to omit the results of such computations because of the uncertainty inherent in them and shall be content to remark that gliadin is much less polar than zein.

Acknowledgment.—We wish to thank J. W. Williams for assistance given in connection with this problem. Grateful acknowledgment is also made to the Wisconsin Alumni Research Foundation and to the University Research Committee for the financial assistance which has made possible the development of these studies.

Summary

1. A combination of the Debye dipole theory and the Perrin study of the hydrodynamics of elongated particles is applied to experimental data involving the protein gliadin in aqueous alcoholic solution.

2. The experimental dispersion of dielectric constant data for dilute gliadin solutions in the frequency range 25,000 to 30,000,000 cycles per second is accounted for quantitatively by the theoretical equations if the molecules are assumed to be ellipsoidal in shape. The values 38,000 for the molecular weight and 8 for the ratio of major to minor axis for the gliadin unit are obtained directly from these data.

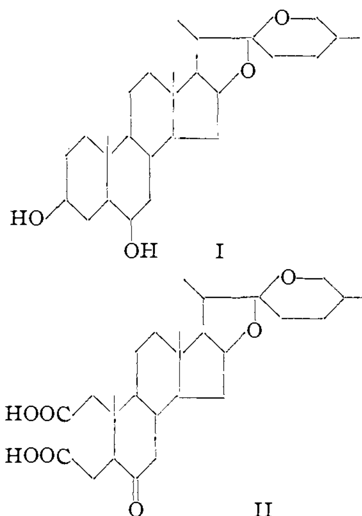
MADISON, WISCONSIN RECEIVED APRIL 24, 1941

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY OF STANFORD UNIVERSITY]

Saponins and Sapogenins. XVIII. The Non-Identity of Chlorogenonic, Digitogenic and Digitoic Acids*

BY C. R. NOLLER AND SEYMOUR LIEBERMAN†

On the basis of extensive work reported by Marker and his co-workers,[1] the two hydroxyl groups of chlorogenin must be located at the 3- and 6-positions of the sterol nucleus (I). Accordingly the ketodibasic acid obtained as one of the products of oxidation of chlorogenin[2] should be identical with digitogenic or digitoic acid (II).



At the time the original preparation of the ketodibasic acid was reported, it was thought that the 25° difference in the melting points of the acids and the 12° difference for the methyl esters was sufficient evidence of non-identity with digitogenic acid. Moreover, digitogenic acid was reported as being readily isomerized by alkali to digitoic acid[3] whereas the ketodibasic acid obtained from chlorogenin was stable to alkali. That it was not identical with digitoic acid was indicated by the 25° difference in melting points of the acids[3] and the 20° difference for the methyl esters.[4]

Marker and Rohrmann[5] have questioned the purity of the oxidation products of digitogenin and have pointed out that no direct comparison was made of digitogenic or digitoic acid with the ketodibasic acid from chlorogenin. We have now made such a comparison[6] and find that the ketodibasic acid from chlorogenin is indeed different from digitogenic acid and from digitoic acid. Although the depressions in melting points of chlorogenonic acid[7] and its dimethyl ester with digito-

* Original manuscript received February 14, 1940.

† Research Assistant on funds from the Rockefeller Foundation.

(1) Marker, Tsukamoto and Turner, THIS JOURNAL, **62**, 2525 (1940); Marker, Jones and Turner, *ibid.*, **62**, 2537 (1940); Marker, Jones, Turner and Rohrmann, *ibid.*, **62**, 3006 (1940); Marker and Turner, *ibid.*, **63**, 767 (1941).

(2) Noller, *ibid.*, **59**, 1092 (1937).

(3) Kiliani, *Arch. Pharm.*, **231**, 448 (1893).

(4) Windaus and Weil, *Z. physiol. Chem.*, **121**, 68 (1922).

(5) Marker and Rohrmann, THIS JOURNAL, **61**, 3479 (1939).

(6) All of the experimental work on the isolation and comparison of the ketoacids and their methyl esters has been repeated independently by both authors. The work on the conversion of chlorogenonic acid to gitogenic acid is that of the junior author alone.

(7) Since the name "chlorogenic acid" is in common use for a well-known depside, the name "chlorogenonic acid" is proposed for the ketodibasic acid from chlorogenin.