increment at high frequencies, $\Delta\epsilon_{\infty}/g$, which depends chiefly upon the volume and hydration of the protein molecules; (3) the critical frequency, ν_c, which depends chiefly upon the size and shape of the molecules and upon the viscosity of the solvent.

Methods are presented for the determination of these quantities by measurements of the dielectric constants of these solutions with a radio frequency bridge. The frequency range covered at present is from 25,000 to 2,500,000 cycles per second, and solutions with specific conductivities up to about 1×10^{-4} mhos/cm. can be studied.

Measurements upon a series of solutions of crystallized carboxyhemoglobin have yielded consistent and reproducible results, and give $\Delta\epsilon_0/g = 0.33$, $\Delta\epsilon_{\infty}/g = -0.11$, and $\nu_c = 1.9 \times 10^6$ cycles/sec., interpreted as indicating a dipole moment for the carboxyhemoglobin molecule of about 500 Debye units, on the basis of an orienting dipole with a molecular weight of 66,700. The method is now being applied to a number of other proteins.

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[CONTRIBUTION FROM THE DEPARTMENT OF PHYSICAL CHEMISTRY, HARVARD MEDICAL SCHOOL, AND THE RESEARCH LABORATORY OF PHYSICAL CHEMISTRY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY, NO. 397]

Studies of the Dielectric Properties of Protein Solutions. II. The Water-Soluble Proteins of Normal Horse Serum[1,2]

BY JOHN D. FERRY AND J. L. ONCLEY

The dielectric constant of a solution of dipolar ions affords an estimate of the dipole moment of the solute, and is an important quantity in the interpretation of the interaction of such a solute with electrolytes and other dipolar ions.[3] Studies of the dielectric constants of solutions of amino acids, peptides, and other dipolar ions of low molecular weight[4] have contributed greatly to knowledge of the physical chemistry of these substances. A method recently described[5] now makes it possible conveniently to measure the dielectric constants of electrolyte-free solutions of proteins over a wide range of frequencies. Data for these multipolar ions of colloidal dimensions may be interpreted by considerations similar to those which hold for the smaller dipolar ions. In addition, from the dispersion of the dielectric constant may be calculated the relaxation time of a protein in solution, which is related to the shape and size of the molecule. Studies with the well-characterized protein, carboxyhemoglobin, already have been reported. The present paper extends this work to certain of the proteins of blood serum.

The protein components of serum usually have been separated by fractional precipitation from concentrated salt solutions. They have been characterized by their chemical compositions, molecular weights, isoelectric points, acid and base binding capacities, and cataphoretic mobilities, and by various physical properties of their solutions.

Albumins.—The albumins of serum are crystallized readily from ammonium sulfate solutions of concentration more than half saturated. Their molecular weight, as determined by osmotic pressure measurements in a variety of solvents, is about 73,000;[6,7] and ultracentrifugal studies, which give almost the same molecular weight, have shown that, whether several times recrystallized or not, they are essentially monodisperse.[8] Although the serum albumins have the same molecular weight, they can be separated by fractional crystallization into portions of widely different solubilities in concentrated salt solutions.[9] The chemical compositions of such fractions also have been shown to differ considerably with respect to content of carbohydrate and certain amino acids.[10]

(1) A preliminary report of this investigation was presented to the Division of Physical and Inorganic Chemistry at the 94th Meeting of the American Chemical Society, September 6–10, 1937.

(2) This investigation has been supported in part by grants from the Committee of the Permanent Charity Fund, Inc., and from the Farnsworth Fund, Harvard Medical School.

(3) Cohn, *Chem. Rev.*, **19**, 241 (1936).

(4) Wyman, *ibid.*, **19**, 213 (1936).

(5) Oncley, THIS JOURNAL, **60**, 1115 (1938).

(6) Adair and Robinson, *Biochem. J.*, **24**, 1864 (1930).

(7) Burk, *J. Biol. Chem.*, **98**, 353 (1932).

(8) (a) Svedberg and Sjögren, THIS JOURNAL, **50**, 3318 (1928); (b) von Mutzenbecher, *Biochem. Z.*, **266**, 250 (1933); (c) McFarlane, *Biochem. J.*, **29**, 407, 660, 1209 (1935).

(9) Sörensen, *Compt. rend. trav. lab. Carlsberg*, **18**, No. 5 (1930).

(10) Hewitt, *Biochem. J.*, **30**, 2229 (1936); **31**, 360 (1937).

Globulins.—None of the proteins of serum which are insoluble in half saturated ammonium sulfate solution has thus far been crystallized. Those which are precipitated by concentrations of ammonium sulfate less than half saturated, and are insoluble in electrolyte-free water, are termed globulins. At least three globulins have recently been separated and characterized by the methods of isoelectric precipitation[11] and cataphoretic mobility.[12] Two of these globulins are only very slightly soluble in such dilute salt solutions (of the order of 10^{-4} *N*) as are at present required for greatest accuracy in measuring the dielectric constants of protein solutions. Investigation of the dielectric properties of these globulins is therefore postponed.

Pseudoglobulin.—If proteins of serum which have been precipitated repeatedly by half saturation with ammonium sulfate are dissolved and subjected to dialysis and electrodialysis, precipitation of globulin occurs, but a water-soluble fraction remains which may be and has sometimes been called "pseudoglobulin." Although soluble in the absence of electrolytes, this "pseudoglobulin" fraction resembles the globulins far more closely than the albumins.

Materials

Normal horse serum[13] was employed in three different preparations of the water-soluble proteins, albumin and pseudoglobulin. The first fractionation consisted in addition to the serum of an equal volume of saturated ammonium sulfate solution, which precipitated all of the globulins.

Albumins.—After filtering off the globulin precipitate, as much of the albumins as possible was crystallized from the filtrate, following the procedure described by Sörensen for crystallizing egg albumin.[14] The crystalline albumins of each preparation were then submitted to fractional crystallization in ammonium sulfate solutions of *p*H of about 5.1, the successive crops of crystals being treated separately. The three different preparations are designated by Roman numerals, and the successive crops of crystals from each preparation by the letters A, B, C, D. Each crop was recrystallized, the least soluble fraction A of preparation II as often as seven times. In the case of IA and IIA, the first crop of crystals was suspended in an ammonium sulfate solution sufficiently dilute to dissolve the more soluble portions before recrystallization. The number of recrystallizations to which each crop was subjected is designated by a subscript.

After recrystallization to the extent indicated, each albumin fraction was dissolved in water, dialyzed to remove salt, concentrated by ultrafiltration when necessary, and electrodialyzed until a specific conductivity of about 10^{-5} ohm^{-1} cm.$^{-1}$ was attained. Protein concentrations were determined by drying aliquots at 105° and weighing the dry protein. Weights obtained in this way are still subject to a correction for moisture, but the magnitude of this correction has not been satisfactorily established, and it is therefore not introduced at present. The *p*H of each solution was measured electrometrically with the hydrogen electrode.

Pseudoglobulin.—Two different procedures were employed in the preparation of the pseudoglobulin.

The globulins of preparation II, which had been precipitated by half saturation with ammonium sulfate, were dissolved in dilute salt and dialyzed against distilled water. Most of the protein was precipitated in this process, and was rejected. The protein now remaining dissolved was reprecipitated by half saturation with ammonium sulfate and redialyzed, the portions remaining dissolved at half saturation of ammonium sulfate (albumin[15]) or precipitated in the absence of salt (globulin) being rejected. The products electrodialyzed after three and four such reprecipitations appeared to have the same dielectric properties, so the process was not continued further. In both cases, the electrodialysis resulted in precipitation of somewhat more protein than could be removed by dialysis alone. The final solutions of "pseudoglobulin" were used for the measurements of dielectric constants.

The globulins of preparation III, having been precipitated by half saturation with ammonium sulfate, were fractionated further before dialysis. Only that fraction soluble in 38% saturated ammonium sulfate was retained. This was precipitated at half saturation with ammonium sulfate; the precipitate was dissolved in dilute salt and reprecipitated at half saturation, then dissolved and dialyzed against distilled water, filtered, and a third time reprecipitated at half saturation of ammonium sulfate. The portions soluble at half saturation of ammonium sulfate were always rejected.[15] The final precipitate was dissolved, dialyzed against distilled water, concentrated by ultrafiltration to about 20% protein, and electrodialyzed. Although no protein precipitated upon electrodialysis, which yielded a solution of conductivity about 10^{-5} and *p*H 5.2, fivefold dilution with conductivity water resulted in the separation of a greenish-blue, viscous gel.[16] Greater dilution caused no further separation, and the solution was reconcentrated by ultrafiltration. The *p*H of this pseudoglobulin was 5.65 at a concentration of 8.0 g. per liter. Further electrodialysis diminished the conductivity somewhat, but did not result in further precipitation or alteration of the dielectric constants of solutions of this fraction. This "pseudoglobulin," which exhibits the same dielectric properties whether prepared by the above method or by that of preparation II, may be constituted of more than one protein.

(11) Green, *J. Biol. Chem.*, **119**, xxxix (1937); THIS JOURNAL, **60**, 1108 (1938).

(12) Tiselius, *Biochem. J.*, **31**, 1464 (1937).

(13) We are much indebted to the Massachusetts Antitoxin Laboratory for the supply of this serum.

(14) Sörensen, *Compt. rend. trav. lab. Carlsberg*, **12**, 12 (1917).

(15) In the initial precipitation of the globulins by half saturation with ammonium sulfate, some albumin is carried down and can in fact be crystallized after re-solution. Most of the albumin may, however, be removed from the "pseudoglobulin" by several reprecipitations.

(16) Addition of water to the gel changed it to a solid precipitate, presumably similar to the P_1 of Green.[11] Dielectric studies on her P_1, P_2, and P_3 will be reported subsequently.

Experimental Results

The bridge method employed for measurements of capacitances and resistances, and the corrections involved in the calculation of the dielectric constant, have been described.[5] All measurements were carried out at 25°. The original capacitance and conductance data over the frequency range from 0.025 to 2.5 megacycles for two albumin solutions are given in Table I. Here κ is the specific conductivity; A the empirical constant in the equation for polarization correction (in units of megohms$^{1/2}$ $\mu\mu f^{-1/2}$); G_1 the initial conductance in parallel with the cell; C^0 the capacitance of the cell when filled with water; ν the frequency in megacycles; G the observed change in conductance (micromhos) upon introduction of the cell containing the protein solution; and C the capacitance reading (in micromicrofarads, $\mu\mu f$) of the standard condenser. The capacitance of the cell, C_x, is calculated from C by equations (9) of the previous paper,[5] using the inductance constants given there. The polarization capacitance correction, ΔC_5, is equal to $AG^2\nu^{-1.5}$. The dielectric constant increment, $\Delta\epsilon$, is given by the relation

$$\Delta\epsilon = (C_x - AG^2\nu^{-1.5} - C^0)/10.95 \qquad (1)$$

where the value of A is chosen so as to make $\Delta\epsilon$ independent of frequency at sufficiently low frequencies. Experimental justification for this procedure already has been given.[5] The polarization correction term, ΔC_5, is proportional to the square of the conductivity. In our studies of each protein, there has been a considerable variation in conductivity and therefore in ΔC_5, but not in the values of $\Delta\epsilon$ at the same protein concentration. Whereas omission of the polarization term in (1) would appreciably increase values of $\Delta\epsilon$ calculated at the lowest frequencies studied—at which the magnitude of $AG^2\nu^{-1.5}$ renders possible the most accurate estimates of A—this term diminishes with increasing frequency, and above one megacycle is entirely negligible.

Albumins.—The data of the dielectric measurements on solutions of two fractions of serum albumin (each once recrystallized) are given in Table I, and the dielectric constant increments are plotted against the logarithm of the frequency in Fig. 1. At the lowest frequencies, $\Delta\epsilon$ is represented as reaching a maximum, much higher for the albumin more soluble in ammonium sulfate (IC_1) than for the less soluble one (IIA_1), though the concentrations of the two solutions are nearly the same. The dispersion of the dielectric increment, however, lies in the same zone for both proteins. Both of these logarithmic dispersion curves are sigmoid and

TABLE I

DIELECTRIC MEASUREMENTS ON SERUM ALBUMIN SOLUTIONS AT 25°

Frequency megacycles, ν	Conductance μmhos, obsd., G	Capacitance Obsd., C	Capacitance Corr., C_x	Polarization corr., $-\Delta C_5$	Dielectric increment, $\Delta\epsilon$
Serum Albumin IIA_1, concentration 19.1 g./liter. $\kappa \times 10^6$ = 11 mhos/cm.; $A \times 10^6$ = 0.1426; G_1 = 5000 μmhos; C^0 = 872 $\mu\mu f$.					
0.025	1346	982	964	65	2.5
.032	1348	962	945	45	2.5
.040	1350	948	931	32	2.4
.050	1352	940	922	23	2.5
.063	1355	933	916	16	2.5
.080	1357	928	910	12	2.4
.100	1362	925	907	8	2.5
.125	1367	922	905	6	2.4
.160	1373	919	901	4	2.3
.200	1382	917	900	3	2.2
.250	1395	915	897	2	2.1
.320	1414	912	893	2	1.8
.400	1440	909	890	1	1.5
.500	1478	907	887	1	1.3
.630	1482	903	882	1	0.9
.800	1530	900	878	0	0.6
1.00	1594	897	874	0	0.1
1.25	1684	896	869	0	—0.3
1.60	1803	896	865	0	−0.7
2.00	1977	902	864	0	−0.7
2.50	2249	911	862	0	−0.9
Serum Albumin IC_1, concentration 21.7 g./liter. $\kappa \times 10^6$ = 12 mhos/cm.; $A \times 10^6$ = 0.140; G_1 = 5000 μmhos; C^0 = 880 $\mu\mu f$.					
0.025	1417	1058	1040	71	8.2
.032	1421	1037	1019	49	8.3
.040	1424	1022	1004	35	8.2
.050	1432	1013	995	26	8.2
.063	1430	1006	988	18	8.3
.080	1434	1000	982	13	8.2
.100	1438	995	977	9	8.1
.125	1446	992	973	7	8.0
.160	1455	989	971	5	7.9
.200	1465	984	966	3	7.6
.250	1485	979	960	3	7.1
.320	1509	975	955	2	6.8
.400	1540	968	948	1	6.2
.500	1585	962	941	1	5.5
.630	1640	954	932	1	4.8
.800	1714	947	923	1	4.0
1.00	1805	941	915	0	3.2
1.25	1928	937	907	0	2.6
1.60	2092	933	898	0	1.7
2.00	2316	934	890	0	1.1
2.50	2673	939	885	0	0.7

nearly symmetrical about the point where ν = 0.85 megacycles.

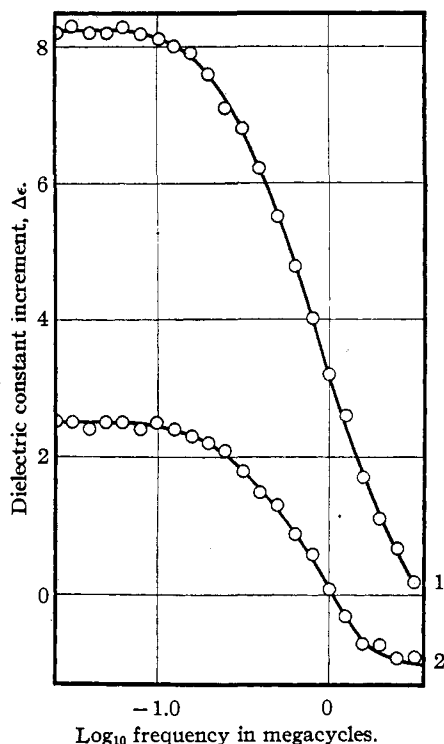

Fig. 1.—Dielectric constant increments of serum albumin solutions plotted against the logarithm of the frequency: 1, albumin IC_1, 21.7 g./liter; 2, albumin IIA_1, 19.1 g./liter.

At the highest frequencies, the dielectric constants of these solutions are lower than that of water, as has been reported by most previous investigators. The measurements do not yet extend to frequencies high enough to estimate values of $\Delta\epsilon_\infty$, which represent the situation where rotation of the solute molecules adds nothing to the dielectric constant and their displacement of water detracts from it.

Measurements upon four other albumin solutions are reported for all frequencies in Table II, in which thec olumns for the uncorrected capacitance C and the polarization correction ΔC_5 are omitted to conserve space, since both are calculable from the equations and constants previously cited. Data on a more concentrated solution of the albumin IIA_1 (*cf.* Table I) are reported in columns 2–4. The other albumins for which data are given have been recrystallized, respectively, two, three, and seven times. The, concentrations of the various solutions are all different. The dispersion curves can be compared readily by adopting the dielectric increment at ν = 0.85 megacycle (which is the inflection point, ν_i, of the curves of Fig. 1) as a reference value, $\Delta\epsilon_i$; and plotting $^1/_2[1 + (\Delta\epsilon - \Delta\epsilon_i)/(\Delta\epsilon_0 - \Delta\epsilon_i)]$ against $\log \nu$. Here $\Delta\epsilon_0$ is taken as the average value of $\Delta\epsilon$ at frequencies of 0.1 megacycle and lower. It is seen (Fig. 2) that within the limits of error all of the measured points fall on the same curve, except for the repeatedly recrystallized preparation IIA_7, which appears to be steeper than the others throughout; though the more purified preparations are all somewhat steeper at high frequencies, where,

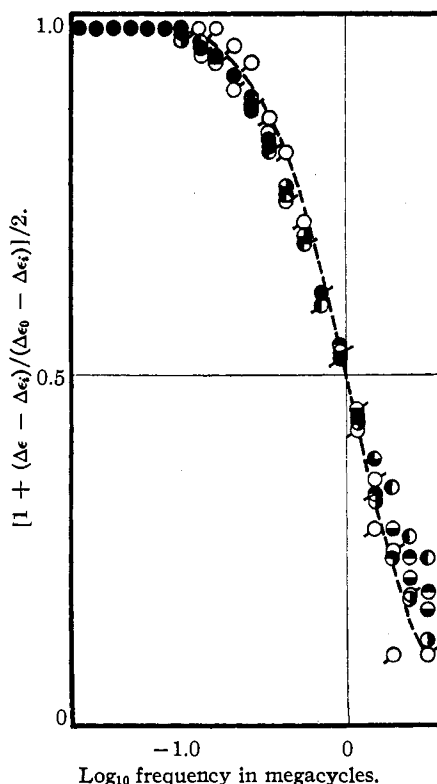

Fig. 2.—Dispersion of the dielectric constant increments of serum albumin solutions: ◓, IIA_1, 19.1 g./liter; ◐, IIA_1, 50.6 g./liter; ◒, IC_1; ◑, IA_2; ♂, $IIIA_3$; ⚲, IIA_7. Filled circles denote coincidences of two or more points.

however, our measurements are the least accurate. These differences are independent of the concentrations of the several solutions, which vary considerably. These dispersion curves, the shapes of which are subsequently considered, represent quantitative descriptions of the dielectric properties of the proteins.

TABLE II

CONDUCTANCES, CAPACITANCES, AND DIELECTRIC CONSTANT INCREMENTS OF SERUM ALBUMIN SOLUTIONS

Temperature 25°; G_1 5000 μmhos

Frequency	Albumin IIA1 50.6 g./liter[a]			Albumin IA2 44.0 g./liter[b]			Albumin IIIA3 28.4 g./liter[c]			Albumin IIA7 31.4 g./liter[d]		
ν	G	C_x	$\Delta\epsilon$	G	C_x	$\Delta\epsilon$	G	C_x	$\Delta\epsilon$	G	C_x	$\Delta\epsilon$
0.025	2387	1126	7.5	1353	996	4.9	1771	962	4.0	2214	1030	3.3
.032	2392	1068	7.1	1355	976	4.9	1772	951	4.2	2217	993	3.3
.040	2398	1034	7.0	1358	964	4.8	1782	942	4.2	2222	967	3.1
.050	2401	1010	7.0	1360	956	4.9	1783	936	4.2	2224	950	3.0
.063	2407	991	6.9	1362	950	5.0	1783	932	4.2	2226	939	3.1
.080	2413	981	7.1	1365	944	4.9	1785	929	4.2	2229	929	3.1
.100	2421	971	7.0	1369	941	4.8	1787	926	4.1	2232	923	3.1
.125	2432	962	6.8	1374	937	4.7	1791	923	4.0	2236	919	3.1
.160	2446	954	6.5	1382	934	4.6	1797	920	3.9	2242	915	3.1
.200	2465	948	6.2	1392	931	4.4	1805	917	3.7	2251	912	3.0
.250	2488	940	5.7	1406	927	4.1	1816	914	3.5	2263	909	2.9
.320	2527	931	5.0	1427	921	3.6	1833	910	3.1	2282	905	2.6
.400	2575	922	4.3	1456	917	3.2	1854	905	2.7	2304	902	2.4
.500	2634	912	3.5	1492	910	2.7	1883	900	2.3	2334	898	2.0
.630	2671	901	2.5	1539	904	2.1	1924	896	1.9	2377	891	1.5
.800	2754	891	1.6	1597	896	1.4	1977	891	1.4	2437	887	1.2
1.00	2858	882	0.9	1667	888	0.7	2042	885	0.9	2511	883	0.8
1.25	2999	873	.1	1757	880	− .1	2127	878	.3	2610	876	.2
1.60	3216	867	− .4	1877	872	− .7	2251	872	− .3	2752	869	− .5
2.00	3496	860	−1.1	2074	867	−1.2	2425	868	− .7	2955	862	−1.1
2.50	3967	858	−1.5	2283	862	−1.6	2692	862	−1.2	3273	857	−1.6

[a] $\kappa \times 10^6 = 20$; $A \times 10^6 = 0.120$; $C^0 = 872$ μμf; $\Delta\epsilon_0 = 7.0$; $\Delta\epsilon_i = 1.4$.
[b] $\kappa \times 10^6 = 11$; $A \times 10^6 = 0.116$; $C^0 = 880$ μμf; $\Delta\epsilon_0 = 4.9$; $\Delta\epsilon_i = 1.2$.
[c] $\kappa \times 10^6 = 14$; $A \times 10^6 = 0.054$; $C^0 = 875$ μμf; $\Delta\epsilon_0 = 4.2$; $\Delta\epsilon_i = 1.2$.
[d] $\kappa \times 10^6 = 18$; $A \times 10^6 = 0.0968$; $C^0 = 874$ μμf; $\Delta\epsilon_0 = 3.1$; $\Delta\epsilon_i = 1.1$.

TABLE III

CONDUCTANCES, CAPACITANCES, AND DIELECTRIC CONSTANT INCREMENTS OF SERUM PSEUDOGLOBULIN SOLUTIONS

Temperature 25°; G_1 5000 μmhos

Frequency	Pseudoglobulin II 15.0 g./liter[a]			8.0 g./liter[b]			Pseudoglobulin III 8.5 g./liter[c]			24.0 g./liter[d]		
ν	G	C_x	$\Delta\epsilon$	G	C_x	$\Delta\epsilon$	G	C_x	$\Delta\epsilon$	G	C_x	$\Delta\epsilon$
0.025	1023	1056	11.6	1319	990	7.1	519	975	7.6	1036	1096	15.8
.032	1025	1041	11.7	1322	978	7.0	521	970	7.6	1041	1084	16.0
.040	1028	1026	11.4	1324	970	6.9	523	965	7.4	1047	1070	15.6
.050	1032	1015	11.1	1328	961	6.6	526	959	7.1	1053	1058	15.1
.063	1037	1005	10.7	1330	955	6.4	529	955	6.8	1060	1047	14.5
.080	1044	993	10.0	1335	947	5.9	533	949	6.4	1072	1034	13.6
.100	1053	983	9.3	1341	941	5.5	540	943	5.9	1085	1020	12.6
.125	1065	972	8.4	1348	934	5.0	547	937	5.4	1101	1006	11.5
.160	1080	959	7.4	1357	927	4.4	555	929	4.7	1121	991	10.2
.200	1097	947	6.4	1369	920	3.9	567	922	4.1	1144	975	8.8
.250	1116	936	5.5	1381	913	3.3	579	915	3.5	1169	960	7.5
.320	1141	924	4.5	1397	906	2.6	594	908	2.9	1201	944	6.1
.400	1165	913	3.5	1413	899	2.0	610	901	2.3	1233	930	4.8
.500	1192	904	2.7	1433	893	1.5	626	896	1.8	1268	917	3.6
.630	1225	894	1.8	1457	889	1.2	627	890	1.3	1308	906	2.7
.800	1261	886	1.1	1485	885	0.8	665	886	0.9	1352	897	1.9
1.00	1301	880	0.6	1518	883	.6	687	882	.6	1402	890	1.2
1.25	1356	875	.1	1564	880	.4	716	880	.4	1467	884	0.7
1.60	1431	869	− .4	1634	876	.0	756	877	.1	1561	879	.3
2.00	1544	866	− .7	1745	875	− .2	818	875	− .1	1684	874	− .2
2.50	1710	862	−1.1	1926	873	− .3	909	873	− .3	1868	870	− .6

[a] Four times reprecipitated. $\kappa \times 10^6 = 8$; $A \times 10^6 = 0.21$; $C^0 = 874$ μμf; $\Delta\epsilon_0 = 11.9$; $\Delta\epsilon_i = 5.7$.
[b] Before final electrodialysis. $\kappa \times 10^6 = 11$; $A \times 10^6 = 0.083$; $C^0 = 876$ μμf; $\Delta\epsilon_0 = 7.1$; $\Delta\epsilon_i = 3.4$.
[c] $\kappa \times 10^6 = 4$; $A \times 10^6 = 0.240$; $C^0 = 876$ μμf; $\Delta\epsilon_0 = 7.7$; $\Delta\epsilon_i = 3.6$.
[d] $\kappa \times 10^6 = 9$; $A \times 10^6 = 0.173$; $C^0 = 876$ μμf; $\Delta\epsilon_0 = 16.3$; $\Delta\epsilon_i = 7.8$.

Pseudoglobulin.—Measurements upon four pseudoglobulin solutions are reported in Table III. Figure 3 shows the dispersion curves, reduced like those of Fig. 2, with 0.24 megacycle as the frequency of the inflection point, ν_i. In this case $\Delta\epsilon_0$ is taken as 2% higher than $\Delta\epsilon$ at 0.032 megacycle, where $\nu/\nu_i = 0.133$, on the basis that the composite curve for the albumins shows $\Delta\epsilon/\Delta\epsilon_0 \cong 0.98$ where $\nu/\nu_i = 0.133$. The polarization correction constant A is at the same time chosen to give the dispersion curve at this point a slope appropriate to $\nu/\nu_i = 0.133$.[17] In the plot of Fig. 3, all the data superimpose within limits of error, regardless of the method of preparation or of the protein concentration. The resulting curve differs from that of serum albumin chiefly in that the dispersion occurs in a much lower frequency range. It is also somewhat less steep than the albumin curve. The curve of Fig. 3 may thus be considered a characteristic description of the protein fraction tentatively denoted "pseudoglobulin."

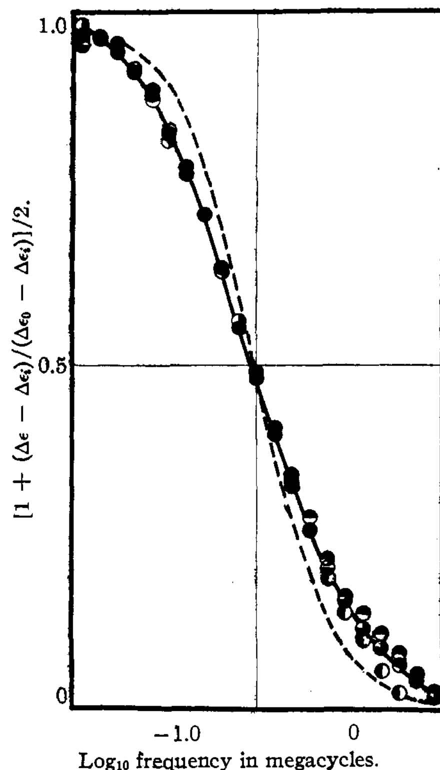

Fig. 3.—Dispersion of the dielectric constant increments of serum pseudoglobulin solutions: ◐, pseudoglobulin II, 4 times reprecipitated, 15.0 g./liter; ◑, III, 8.0 g./liter; ◒, III, 8.5 g./liter; ◓, III, 24.0 g./liter. Filled circles denote coincidences of two or more points.

(17) The uncertainty involved in this choice is of small consequence. Development of the apparatus for measurements at lower frequencies will obviate such slight uncertainties in obtaining $\Delta\epsilon_0$ when dispersion occurs in this region.

Low Frequency Dielectric Increments

The influence of proteins upon the static dielectric constants of solutions is given by the measurements at low frequencies below the zone of anomalous dispersion. The values for the low frequency dielectric increment, $\Delta\epsilon_0$, are believed to be accurate to within 0.3 unit, and, as do the dispersion curves, serve to characterize proteins. Furthermore, as our studies on the serum albumins demonstrate, proteins with the same dispersion curves may have quite different dielectric constant increments (Fig. 1).

Albumins.—Values of $\Delta\epsilon_0$ estimated for the albumin solutions heretofore cited, as well as for certain others, data for the dispersion curves of which are not reported (since they are similar to those in Tables I and II), are given at a number of concentrations in Table IV. The *p*H and the conductivity of each solution are also reported. Although the dispersion curves of many of these solutions were closely similar, as noted, their dielectric increments are very different. Values of $\Delta\epsilon_0$ for four albumin preparations are plotted against the concentration in Fig. 4 (curves 2–5). Over the range investigated, it appears that, as with hemoglobin,[5] and with amino acids and peptides,[3] the dielectric constant increases linearly with the concentration. The slope of

TABLE IV

LOW FREQUENCY DIELECTRIC CONSTANT INCREMENTS OF ALBUMIN SOLUTIONS

Albumin	Concn., g./liter	*p*H	$\kappa \times 10^6$	$\Delta\epsilon_0$	$\Delta\epsilon_0/g$
IIA_1	19.1	5.12	11	2.5	0.13
	50.6	4.93	20	7.0	.14
IA_2	13.0	4.98	7	1.7	.13
	26.2	4.89	9	3.3	.13
	44.0	4.82	11	4.9	.11
$IIIA_3$	15.2	4.93	13	2.7	.18
	24.3	..	13	2.9	.12
	28.4	..	14	4.2	.15
	51.4	4.81	17	7.1	.14
IIA_7	31.4	4.80	18	3.1	.10
	66.2	4.68	22	5.0	.08
IC_1	11.8	5.40	5	4.7	.40
	21.7	5.20	12	8.2	.38
$IIID_2$	6.8	5.22	17	1.7	.25
	12.3	..	24	3.4	.28
	26.7	5.17	36	7.7	.29
	49.1	5.07	52	14.3	.29

each line in Fig. 4 represents the quantity $\Delta\epsilon_0/g$, which for each albumin studied is apparently independent of concentration, and is much greater for those fractions more soluble in ammonium sulfate than for those less soluble.

A few measurements of solubility in ammonium sulfate solutions (pH about 5.1) were made on the fractions IC_1 and IA_2. The salting-out curves, following the equation of Cohn,[18] $\log S = \beta - K'_s\Gamma/2$, where S is the solubility of protein in g. per liter, and $\Gamma/2$ the ionic strength of ammonium sulfate per liter, may be described by the following constants: IC_1, $\beta = 3.9$, $K'_s = 0.65$; IA_2, $\beta = 5.6$, $K'_s = 1.11$. The dielectric increment per g. per liter, $\Delta\epsilon_0/g$, is 0.39 for albumin IC_1, which is about five times as soluble in ammonium sulfate solutions as albumin IA_2 with $\Delta\epsilon_0/g$ equal to 0.12. In another preparation, six recrystallizations of a fraction of low solubility reduced the value of $\Delta\epsilon_0/g$ from 0.14 (IIA_1) to 0.10 (IIA_7). The data of Table IV include, besides the extreme cases illustrated in Fig. 4, fractions of intermediate dielectric properties.

Pseudoglobulin.—Values of $\Delta\epsilon_0$ estimated for the pseudoglobulin solutions heretofore cited, as well as for a number of others, are given in Table V, and are plotted as curve 1 in Fig. 4. In contrast to serum albumin—which is crystalline and from some points of view apparently monodisperse, yet different preparations of which vary widely in solubility in salt solutions and in dielectric increment—the pseudoglobulin, which in some respects is not so well characterized a protein, appears to have much the same value of $\Delta\epsilon_0$ at the same concentration, whether prepared as in preparation II or III. Moreover, the influence of this fraction on the dielectric constant is much greater than that of the albumins. Unlike the case of hemoglobin and the serum albumins, the curve for $\Delta\epsilon_0$ against concentration (Fig. 4) indicates that the $\Delta\epsilon_0/g$ of pseudoglobulin is nowhere independent of concentration. The value of $\Delta\epsilon_0/g$ at infinite dilution cannot be estimated with great accuracy, but it is approximately 1.1 per g. per liter, which is over ten times that of the least soluble serum albumin. Whether this fraction is monodisperse and what its molecular weight is remain to be investigated. It is possible that the constants reported for it may change somewhat with further purification.

TABLE V

LOW FREQUENCY DIELECTRIC CONSTANT INCREMENTS OF PSEUDOGLOBULIN SOLUTIONS

Pseudo-globulin	Concn., g./liter	pH	$\kappa \times 10^6$	$\Delta\epsilon_0$	$\Delta\epsilon_0/g$
II, 3 times	8.2	5.69	14	7.7	0.94
repptd.	17.6	5.70	26	13.2	.75
	32.0	5.77	43	19.6	.61
II, 4 times	4.4	..	6	3.7	.84
repptd.	8.2	5.73	6	7.7	.94
	15.0	5.64	8	11.9	.79
	28.0	5.73	12	19.1	.68
III	8.0[a]	5.65	11	7.1	.89
	8.5	5.43	4	7.7	.91
	24.0	5.43	9	16.3	.68

[a] Before final electrodialysis.

(18) Cohn, *Physiol. Rev.*, **5**, 349 (1925).

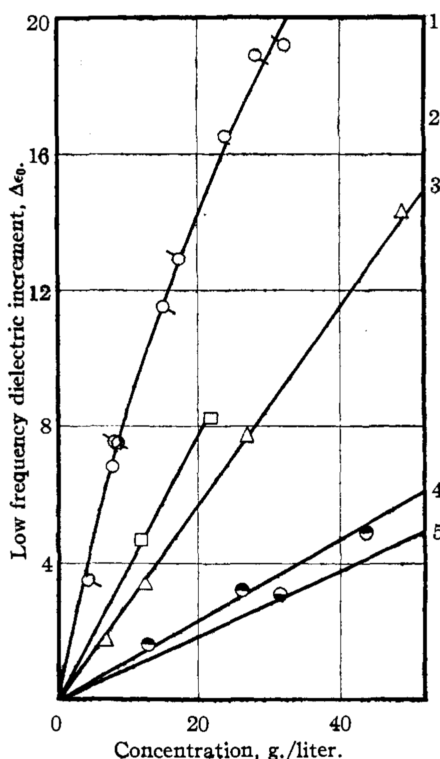

Fig. 4.—Low frequency dielectric constant increments. 1, Pseudoglobulin: ♂, II, 3 times repptd.; ♀, II, 4 times repptd.; O, III. 2, Albumin IC_1. 3, Albumin $IIID_2$. 4, Albumin IA_2. 5, Albumin IIA_7.

The dielectric increments per gram of the proteins thus far studied (0.10 for serum albumin IIA_7 to 1.1 for serum pseudoglobulin) cover a similar range to that of the amino acids and peptides (0.17 for leucine to 1.3 for lysyl-glutamic acid). The dielectric increments of the proteins per mole are, of course, very much greater than those of the smaller dipolar ions. Taking the molecular weights for the proteins as 73,000 and 150,000, respectively,[6,7,21] the dielectric increments just

quoted are per mole about 7000 for the albumin and 160,000 for the pseudoglobulin, as contrasted with 23 for leucine and 345 for lysyl-glutamic acid.

Discussion

Dipole Moments.—An estimate of the dipole moments of these molecules may be made from the empirical equation[5]

$$\mu = 2.9\sqrt{M(\Delta\epsilon_0 - \Delta\epsilon_\infty)/g}$$

where M is the molecular weight. We shall assume $\Delta\epsilon_\infty = 2\Delta\epsilon_i - \Delta\epsilon_0$ (see below).

For the serum albumins, the values are low or high according to the dielectric increments. For the albumin of lowest polarity IIA_7, $(\Delta\epsilon_0 - \Delta\epsilon_\infty)/g = 0.13$ and $\mu = 280$ Debye units. For the most polar albumin, IC_1, $(\Delta\epsilon_0 - \Delta\epsilon_\infty)/g = 0.42$ and $\mu = 510$ Debye units.

In the case of the serum pseudoglobulin, the ratio $(\Delta\epsilon_0 - \Delta\epsilon_\infty)/g$ is not independent of concentration. Its value at infinite dilution, as in the case of $\Delta\epsilon_0/g$ (1.1 per g./liter), can be estimated only tentatively. With the extrapolated value for $(\Delta\epsilon_0 - \Delta\epsilon_\infty)/g$ of 1.2 per g./liter, $\mu = 1200$ Debye units.

The dipole moments calculated for the two albumins of most widely different solubilities in salt solutions thus differ by a factor of about 1.8, whereas all the pseudoglobulin preparations have the same average moment, which is much higher than that of either albumin. While the values estimated may be somewhat too large, they are probably much nearer the truth than figures calculated from the ordinary Debye formula,[19] which is not valid for solutions in polar solvents.

Dispersion.—The theory of the anomalous dispersion of the dielectric constants of polar liquids was developed by Debye[19] (pp. 77 ff.); it already has been discussed in connection with protein solutions.[3,20] In the first paper of this series,[5] the dispersion for hemoglobin has been shown to follow the equation of Debye

$$\Delta\epsilon = \Delta\epsilon_0 - \frac{(\Delta\epsilon_0 - \Delta\epsilon_\infty)\,\nu^2/\nu_c^2}{1 + \nu^2/\nu_c^2} \qquad (2)$$

where ν_c is the critical frequency. When ν is plotted logarithmically, the coördinates of the inflection point are $\log \nu_i = \log \nu_c$ and $\Delta\epsilon_i = (\Delta\epsilon_0 + \Delta\epsilon_\infty)/2$. The form of this function is shown in Figs. 2 and 3 by dashed lines, drawn in each case by obtaining ν_c and $\Delta\epsilon_\infty (=2\Delta\epsilon_i - \Delta\epsilon_0)$ from the experimental inflection point. If the dispersion for serum albumin and pseudoglobulin followed the Debye equation, the experimental points should fall on the calculated curves throughout, instead of only at ν_i where they are made to coincide. The deviations are actually small, though significant at least for the pseudoglobulin. In any case, the reproducibility of the curves renders them very valuable for characterization of these proteins.

Deviation from the theoretical curves might indicate that the simple Debye treatment is inapplicable to solutions of some proteins, even those composed of a single, symmetrical molecular species. For example, interaction between solute molecules with hindering of rotation might have the effect of spreading the critical frequency over a continuous range of values, which could produce slightly flattened dispersion curves following the experimental points of Figs. 2 and 3. On the other hand, the observed dispersion can be fitted by adding two or more components, each of which follows the Debye equation, so that

$$\Delta\epsilon = \Delta\epsilon_0 - \frac{\Delta\epsilon_1\,\nu^2/\nu_{c(1)}^2}{1 + \nu^2/\nu_{c(1)}^2} - \frac{\Delta\epsilon_2\,\nu^2/\nu_{c(2)}^2}{1 + \nu^2/\nu_{c(2)}^2} - \dots \qquad (3)$$

In this type of complex dispersion, the several distinct critical frequencies, $\nu_{c(1)}, \nu_{c(2)} \dots$ might be ascribed either to rotation of the same asymmetrical molecule about different axes, or to the presence of two or more symmetrical molecular species. In the former case, analysis of the dispersion curves should yield information about not only the electrical but also the spatial symmetry of the molecule; the angles between its dipole moment and its geometrical axes could be calculated. If more than one molecular species be present, the shape of the curve should depend upon physical chemical conditions (in the case of a reversible equilibrium) or upon the state of purification (in the case of a mixture of two unrelated proteins).

The pseudoglobulin curve (Fig. 3) can be represented on the basis of equation (3) by two dispersion terms, with $\Delta\epsilon_1 = \Delta\epsilon_i$, $\Delta\epsilon_2 = 2\Delta\epsilon_i - \Delta\epsilon_0$, $\nu_{c(1)} = 0.12$ megacycle, and $\nu_{c(2)} = 0.48$ megacycle; *i. e.*, two Debye curves of approximately equal height, with one critical frequency equal to twice the observed inflection frequency and the other equal to half the latter.

The albumin data can be treated similarly, though in this case, since the most often recrystallized preparation appears to conform to the theoretical curve rather more closely than the others,

(19) Debye, "Polar Molecules," Chemical Catalog Co., New York, 1929, p. 36.

(20) Williams, *Trans. Faraday Soc.*, **30**, 723 (1934).

there remains the possibility that further purification may yield a product whose dispersion shows no deviation from the simple Debye theory. Experiments to determine homogeneity from this point of view are in progress.

Relaxation Times.—Serum albumin is supposed to be monodisperse from the point of view of molecular weight[8] and electrical mobility.[12] Recently reported values for the molecular weight range from 67,000 by the sedimentation velocity method[21] to 74,600 by the osmotic pressure method.[7] On the basis of the estimate[22] of 0.81 as the specific volume of isoelectric hydrated serum albumin crystals, the molecular volume of protein hydrate would not be greater than 80,000, as compared with 50,000 calculated from the lower molecular weight with the apparent specific volume of 0.748[8a] for anhydrous protein in solution. Were this molecule symmetrical, the relaxation time in water at 25° (given by $\tau = 3\eta V/RT$, where η is the viscosity and V the molecular volume) should be 0.055×10^{-6} sec., or at a maximum 0.088×10^{-6}. These values are only about a third of the figure 0.19×10^{-6} sec. obtained from our measurements, taking as ν_c ($= 1/2\pi\tau$) the inflection frequency of Fig. 2.

Serum globulin is divisible into a number of fractions differing in isoelectric points,[11,12] solubility,[11] electrical mobility,[12] and viscosity.[23] The pseudoglobulin fraction prepared by the methods described above is being characterized further by all these methods. Despite the diversity in these chemical properties, the molecular weight of the main component of serum globulin has been given as 150,000 by ultracentrifugal studies[21] and 175,000[6] and 177,000[24] by measurements of osmotic pressure; while the ultracentrifuge also indicates a small component of molecular weight about 400,000. Relaxation times calculated from the smallest and largest of these values (in the absence of information concerning the molecular weight of the "pseudoglobulin" we have used) would be 0.12×10^{-6} and 0.32×10^{-6} sec.[25] From our measurements, on the other hand, we have from the inflection frequency of Fig. 3 $\tau = 0.66 \times 10^{-6}$ sec.; or, from the critical frequencies of the two Debye curves which are fitted to the data, $\tau_1 = 1.3 \times 10^{-6}$ and $\tau_2 = 0.33 \times 10^{-6}$. The discrepancies between the relaxation times calculated from the molecular weights and those observed are rather greater than in the case of the albumin (except that the τ_2 from the complex analysis corresponds fairly well to that calculated for the species of 400,000); whereas for hemoglobin the agreement appears to be satisfactory.[5]

While the dispersion of the dielectric constant, as reported, is an adequate quantitative description of each protein studied, the theory as thus far developed evidently does not permit calculation of molecular weights from observed critical frequencies. The extent to which asymmetry may account for not only the shape of the dispersion curves, but also their position at lower frequencies than anticipated, is being further investigated. In this connection it may be noted that the viscosities of solutions of hemoglobin, serum albumin, and serum pseudoglobulin deviate from the prediction of the Einstein equation increasingly in the order named[26]—which is also the order of increasing deviation from the Debye equation, as regards both shape and location of dispersion curves.

We are deeply indebted to Professor Edwin J. Cohn for his generous aid and indispensable advice throughout this investigation.

Summary

1. The dielectric constants of solutions of the pseudoglobulin and several crystalline fractions of albumin from normal horse serum have been measured over a frequency range of 25,000 to 2,500,000 cycles.

2. All these proteins increase the static dielectric constant of water.

3. The dielectric constant increments of those serum albumins more soluble in ammonium sulfate solutions are greater than those of the less soluble; the dielectric constant increment of the pseudoglobulin is much greater still.

4. The dielectric constant increments per gram are of the same order of magnitude for these proteins as for the amino acids, and range from 0.1 for the least polar albumin to 1.1 for the pseudoglobulin.

5. The dielectric increments per mole range from 7000 for the least polar albumin to 160,000 for the pseudoglobulin.

(21) Svedberg, *Chem. Rev.*, **20**, 81 (1937); von Mutzenbecher and Svedberg, *Naturwissenschaften*, **21**, 331 (1933).

(22) Adair and Adair, *Proc. Roy. Soc.* (London), **B120**, 422 (1936).

(23) Unpublished experiments by A. A. Green and K. Fahey.

(24) Burk, *J. Biol. Chem.*, **121**, 373 (1937).

(25) Adair and Adair[22] give no value for the specific volume of hydrated pseudoglobulin. Their value for euglobulin is 0.81; although there is no justification for using this, it would increase the calculated values of τ by about 40%.

(26) Daniel and Cohn, THIS JOURNAL, **58**, 415 (1936).

6. The frequency dependence of the dielectric constant increment is the same for all the albumins, deviating only slightly from a theoretical Debye curve with a critical frequency of 0.85 megacycle. The dispersion of the dielectric increment of all the pseudoglobulin solutions is given by a single curve which has a small but significant deviation from a Debye curve with a critical frequency of 0.24 megacycle.

7. The relaxation times calculated from these critical frequencies are several times larger than would be calculated from the molecular weights on the basis of present theories.

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[CONTRIBUTION FROM THE FRUIT PRODUCTS DIVISION, UNIVERSITY OF CALIFORNIA]

The Rate of Conversion of Chlorophyll to Pheophytin

BY M. A. JOSLYN AND G. MACKINNEY

The conversion of chlorophyll to pheophytin, in dilute acid solutions, resulting in the replacement of magnesium with hydrogen, is a reaction which occurs at a measurable rate in acetone solutions. Information on the rate of this reaction is of interest because of possible contributions to an understanding of the complex linkage of magnesium in the phorbin nucleus. Willstätter and Stoll[1] have shown that colloidal solutions of chlorophyll in water may be decomposed by carbon dioxide, and further that chlorophyll *a* is more rapidly converted to pheophytin *a* than the *b* component to the corresponding pheophytin *b*. Under comparable conditions for measuring the uptake of carbon dioxide, they observed, in forty-eight hours, 25 per cent. decomposition of chlorophyll *b*, as against 80% for chlorophyll *a* in twenty-four hours. These results were obtained by analysis of the magnesium contents of the partially decomposed chlorophylls.

Dorries[2] and Röben and Dorries[3] have utilized the appearance in the pheophytin spectrum of a prominent band (which has a maximum absorption close to 5350 Å. in acetone solution) as a qualitative means of detecting injury to leaves exposed to various gases. By observing spectrophotometrically the increased absorption at this wave length, we have estimated the proportion of chlorophyll converted to pheophytin in solutions of various acids in 90% aqueous acetone, at given time intervals.

Our data substantiate the validity of this calculation, where oxalic acid was used, because the initial rate was maintained for several hours, and we were able to demonstrate one chlorophyll and one pheophytin component by the Tswett column technique, in spite of the difficulties attendant upon the separation of components in solutions necessarily dilute for spectroscopic analysis. No other products were detected, so that the course of this reaction was reasonably established. Measurements were also made with sulfurous, sulfuric, and hydrochloric acids. The reaction rate was too slow with acetic acid for accurate measurement. The data may be interpreted on the basis of a first order reaction with respect to equivalent concentration of acid (*i. e.*, normality). The data are less conclusive with regard to chlorophyll concentration. They favor a second order reaction for chlorophyll in oxalic acid solution, but with the other acids the initial rate is not maintained for a sufficient time for a satisfactory decision.

Experimental

Stock solutions of chlorophyll were prepared by dissolving known amounts of chlorophyll (5X grade, American Chlorophyll Company) in acetone. Only preparations which responded satisfactorily to the tests listed by Willstätter and Stoll,[4] including the Molisch phase test, were used. Aliquots were pipetted into 250-ml. volumetric flasks, diluted to *ca.* 200 ml. with acetone, the appropriate amount of water and standardized acid added, and the solutions quickly brought to volume with acetone. The solvent was 90% aqueous acetone. The acid concentrations were varied from 0.05 to 0.0001 *N*, and the chlorophyll concentrations from 13.3 to 66.4 mg. per liter. The sulfurous acid solution was freshly prepared from liquid sulfur dioxide. Solutions were stored in the dark at room temperature (20–25°), and periodic measurements were made.

A Bausch and Lomb Universal Spectrophotometer was used to estimate visually the transmissions at the position

(1) Willstätter and Stoll, "Assimilation der Kohlensäure," Verlag von Julius Springer, Berlin, 1918.

(2) Dorries, *Ber. bot. Ges.*, **50b**, 47 (1932).

(3) Röben and Dorries, *ibid.*, **50b**, 52 (1932).

(4) Willstätter and Stoll, "Untersuchungen über Chlorophyll," Verlag von Julius Springer, Berlin, 1913, p. 144.