and

$$\int \frac{c\,dx}{[a+x][b+x]^2} + \int \frac{x\,dx}{[a+x][b+x]^2} = kt + C \quad \text{(VI)}$$

whence a straight line should be obtained by plotting the sum of the integrals on the left side of the equation against time. In Fig. 2 it will be observed that reasonably straight lines were obtained throughout at least 54% of reaction[13] and that the rate constants of the different runs are in fair agreement in spite of the fact that no correction could be made for the change in the nature of the reaction medium brought about by a fourfold change in anilinium ion concentration.[14]

While there may be other mechanisms which could be postulated to fit the data, it is interesting that a mechanism based upon the intermediate formation of a sulfur trioxide addition complex with aniline[1] is in agreement with equation IV. Thus, if the over-all transformation of aniline to sulfanilic acid can be represented by the equations VII to IX

$$2H_2SO_4 \overset{K_1}{\rightleftharpoons} SO_3 + OH_3^+ + HSO_4^- \quad \text{(VII)}$$

$$SO_3 + C_6H_5NH_3^+ \overset{K_2}{\rightleftharpoons} C_6H_5NHSO_3H + H^+ \quad \text{(VIII)}$$

$$C_6H_5NHSO_3H + H_2SO_4 \xrightarrow{k_3} [HO_3SC_6H_4NHSO_3H + H_2O] \downarrow$$

$$HO_3SC_6H_4NH_2 + H_2SO_4 \quad \text{(IX)}$$

(13) The last two points of run c in Fig. 2 represent 65 and 70% reaction, respectively.

(14) Neither anhydrous sodium sulfate nor anhydrous potassium sulfate were found to be soluble enough in 96% sulfuric acid to be used to maintain a constant ionic strength.

it can be seen that

$$\frac{dP}{dt} = k_3[C_6H_5NHSO_3H][H_2SO_4]$$

whence

$$\frac{dP}{dt} = k_3K_2\frac{[SO_3][C_6H_5NH_3^+][H_2SO_4]}{[H^+]} = k_3K_1K_2\frac{[C_6H_5NH_3^+][H_2SO_4]^2}{[H^+][HSO_4^-][OH_3^+]} \quad \text{(XI)}$$

and since

$$K_{H_2SO_4} = \frac{[H^+][HSO_4^-]}{H_2SO_4} \quad \text{(XII)}$$

$$\frac{dP}{dt} = \frac{k_3K_1K_2}{K_{H_2SO_4}}\frac{[C_6H_5NH_3^+][H_2SO_4]^2}{[OH_3^+]} \quad \text{(XIII)}$$

Acknowledgment.—The author wishes to express his appreciation to the trustees of the Frank B. Jewett Fellowships for a fellowship which made part of this investigation possible.

Summary

A study of the influence of sulfuric acid on the sulfonation of aniline and dimethylaniline at 140 and 185° has shown that in spite of the similarity of the starting materials, the conditions for reaction, and the isomers obtained, the mechanisms of the reactions are different. At 185° when the reaction is carried out *in vacuo* the rate of reaction is increased in the case of aniline and decreased in the case of dimethylaniline by the addition of excess sulfuric acid. At 140° when the reaction is carried out in a closed system the rate of sulfonation of aniline appears to follow the equation

$$\frac{dP}{dt} = \frac{k[C_6H_5NH_3^+][H_2SO_4]^2}{[OH_3^+]}$$

Reaction mechanisms have been proposed which are compatible with these facts.

URBANA, ILLINOIS RECEIVED FEBRUARY 10, 1947

[CONTRIBUTION FROM THE DEPARTMENTS OF CHEMISTRY AND BIOLOGICAL CHEMISTRY OF WASHINGTON UNIVERSITY]

Zein Solutions as Association–Dissociation Systems[1]

BY BARRETT L. SCALLET[2]

Sørensen[3] in 1930 advanced the hypothesis that the molecules of soluble proteins consist of a number of components comparatively loosely held together by means of secondary valences. The amino acid residues within these components are of course linked together by principal valences, but the secondary linkages are so much weaker that changes in the salt content, hydrogen ion activity, alcohol content, or temperature of a protein solution may give rise to reversible dissociation.

(1) From a dissertation presented to the Board of Graduate Studies of Washington University in partial fulfilment of the requirements for the degree of Doctor of Philosophy, June, 1946.

(2) Present address: Corn Products Division, Anheuser-Busch, Inc., St. Louis, Missouri.

(3) Sørensen, *Compt. rend. trav. lab. Carlsberg*, **18**, (no. 5), 1 (1930).

Sørensen's conclusions were based on solubility relationships, amino acid analyses, osmotic pressure measurements, acid and base titrations and optical rotation measurements on various proteins, especially serum albumin. Eriksson-Quensel and Svedberg[4] showed clearly by means of the ultracentrifuge that the hemocyanins dissociate reversibly within certain *p*H ranges.

Sinclair and Gortner[5] demonstrated that gliadin must be considered to be a reversible association–dissociation system, since fractionation followed by reworking of the fractions yielded two products identical in physical properties with the original gliadin preparation.

(4) Eriksson-Quensel and Svedberg, *Biol. Bull.*, **71**, 498 (1936).

(5) Sinclair and Gortner, *Cereal Chem.*, **10**, 171 (1933).

Watson,[6] Watson, Williams and Arrhenius,[7] and Gortner and McDonald[8] have shown that zein solutions are polydisperse. These investigators have separated zein into fractions which are stable for long periods of time and which exhibit pronounced differences in sedimentation and diffusion rates, osmotic pressure, and optical rotation. They are of the opinion that the zein components do not form an association–dissociation system, but exist as entities unaltered by small changes in the solvent. The present electrophoresis experiments cannot furnish information on molecular size, but they indicate that solutions of zein and of its fractions form sensitive association–dissociation systems, and that the equilibrium between components can be shifted readily by alterations in protein concentration, salt concentration or *p*H.

Effect of Protein and Buffer Concentration.—Electrophoretic patterns of zein solutions identical except for protein concentration show that zein dissociates in dilute solution. Figure 1 shows the patterns obtained for fraction 10-A-35[9] at concentrations ranging from 0.15 to 1.3 g./100 ml. Two-hour pictures are given here since the separation into slow-moving and fast-moving peaks shows up especially well in the early stages of electrophoresis.[10] Measurements of the areas under these curves by the method of Tiselius and Kabat[11] indicate that at protein concentrations of 1.3, 0.6 and 0.3 g./100 ml. the proportions of fast-moving components are 31, 50 and 71%, respectively. Further decrease in protein concentration to 0.15 g./100 ml. does not alter the proportions of fast- and slow-moving components.

In Fig. 2 are shown later pictures in the same runs; in dilute solution (0.3 g./100 ml.) at least six zein components of different electrophoretic mobility appear (2a). Figure 2b shows that in more concentrated solution (1.3 g./100 ml.) only three or four components appear. The remaining ones are undoubtedly tied up in the slow-moving portion. Figures 2c and 2d show the effect of increasing the buffer concentration (from 0.1 *M*) to 0.5 and 1.0 *M*, respectively. In both cases the components have associated into a single molecule which does not break down even on prolonged electrophoresis.

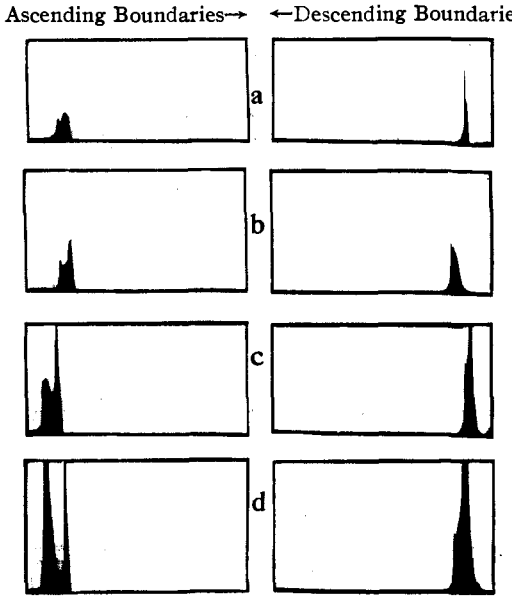

Fig. 1.—Electrophoretic patterns showing the effect of protein concentration on degree of association of zein fraction 10-A-35: a, 0.15 g./100 ml.; b, 0.3 g./100 ml.; c, 0.6 g./100 ml.; d, 1.3 g./100 ml.; acetate buffer (*p*H 5.4, μ 0.016) in 60% alcohol; time, 2 hours at 315 volts and 1.5 milliamperes; potential drop, 9.6 volts per cm.; temperature, 2–3°.

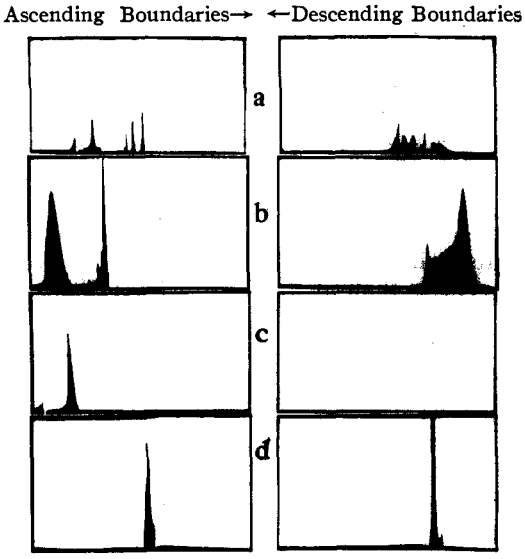

Fig. 2.—Electrophoretic patterns showing the effect of protein concentration and buffer concentration: a, 0.3 g./100 ml., fraction 10-A-35, 10.5 hours; b, 1.3 g./100 ml., fraction 10-A-35, 5.8 hours; c, 1 g./100 ml., preparation 1 in 0.5 *M* acetate buffer, μ 0.56, *p*H 6.8, 10.4 hours (descending boundary disrupted); d, 1 g./100 ml., preparation 1 in *M* acetate buffer, *p*H 5.4, μ 0.16, 14.4 hours.

Effect of *p*H.—Electrophoresis of zein was studied over a wide range of *p*H at constant ionic strength. Patterns are given in Fig. 3. At *p*H 4.6 there is very little shift in the equilibrium between fast-moving and slow-moving com-

(6) Watson, Dissertation, University of Wisconsin, 1938.

(7) Watson, Williams and Arrhenius, *Nature*, **137**, 322 (1936).

(8) Gortner and McDonald, *Cereal Chem.*, **21**, 324 (1944).

(9) In code designations of zein fractions, the first number is the serial number of the fractionation, the letter gives the number of times the fraction has been precipitated, and the final number gives the percentage of alcohol from which the sample was precipitated (see Experimental).

(10) Ascending and descending electrophoretic patterns generally differ, since the ascending boundary is moving into buffer solution, while the descending boundary is moving into protein solution. In the case of zein, dissociation causes a large difference. This question is considered more fully in the Discussion.

(11) Tiselius and Kabat, *J. Exptl. Med.*, **69**, 119 (1939).

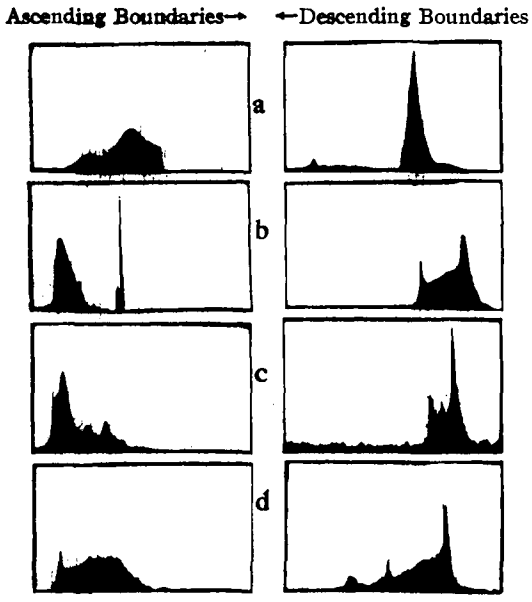

Fig. 3.—Electrophoretic patterns showing the effect of *p*H on fraction 10-A-35 at a concentration of 1 g./100 ml.: a, chloroacetate buffer, *p*H 3.6, 11.7 hours at 210 volts; b, lactate buffer, *p*H 4.6, 5.9 hours at 315 volts; c, succinate buffer, *p*H 8.2, 11.3 hours; d, veronal buffer, *p*H 10.0, 8.3 hours; for *p*H 5.4, see Fig. 2b. All buffers are in 60% alcohol, μ 0.016.

ponents as compared with the equilibrium at *p*H 5.4 (Fig. 2b). At *p*H 3.6 the aggregate breaks down almost entirely into fast-moving components, while at *p*H 8.2 dissociation is only a little greater than at *p*H 5.4. At *p*H 10.0, however, dissociation is very extensive and, judging by the large number of peaks obtained, probably irreversible.

The *p*H–mobility diagrams of the slow-moving and fast-moving components are given in Fig. 4. The isoelectric point of the slow-moving portion is at *p*H 5.8, and the fastest-moving component has an isoelectric point at *p*H 7.5.

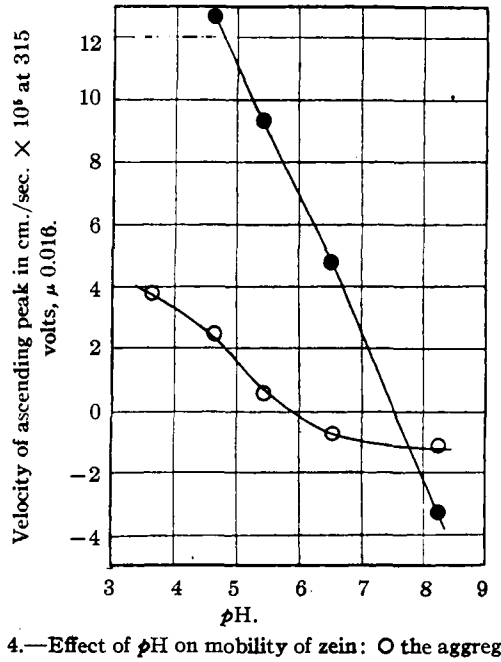

Fig. 4.—Effect of *p*H on mobility of zein: O the aggregate; ● fastest component.

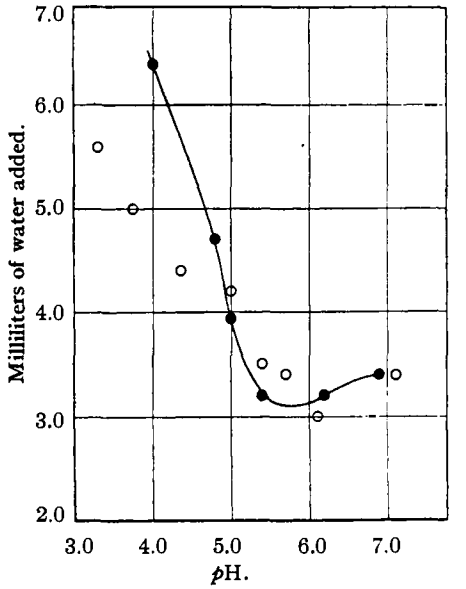

Fig. 5.—Critical precipitation curves for unfractionated zein in acetate buffers, ●, and in lactate buffers O.

The isoelectric point of whole zein was determined by adding water to 1% solutions of zein in alcoholic acetate and lactate buffers of the same ionic strength but different *p*H. The volumes of water required to produce cloudiness are plotted against *p*H in Fig. 5. The curve for acetate buffers indicates an isoelectric point of 5.8, while

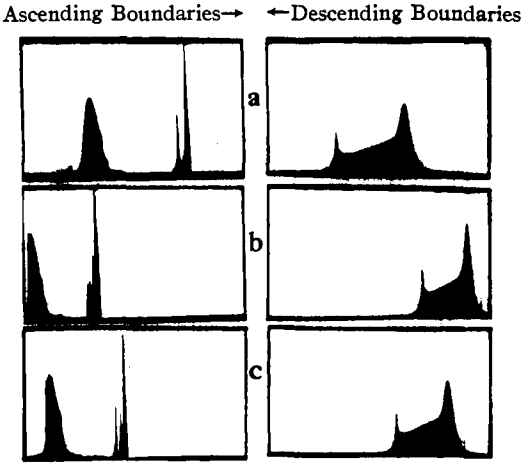

Fig. 6.—Electrophoretic patterns of zein preparation 2 and its fractions: a, whole zein, 9.0 hours; b, fraction precipitated from 49% alcohol, 7.1 hours; c, fraction precipitated from 45% alcohol, 7.7 hours; all concentrations 1.5 g./100 ml. in 60% alcoholic acetate buffer, *p*H 5.4.

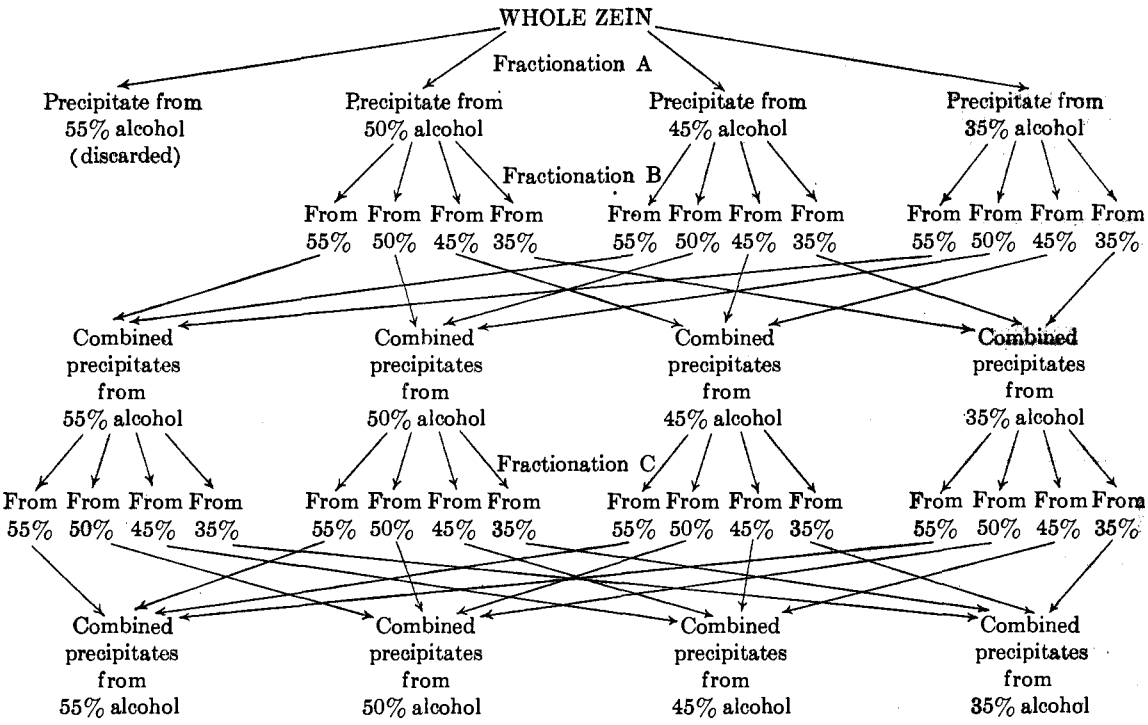

Fig. 7.—Multiple fractionation scheme for zein.

the erratic data for lactate buffers indicate a somewhat higher value.

Fractionation Studies.—Because of the great amount of interaction between components in solution, it was not possible to separate them by fractional precipitation. Figure 6 shows the electrophoretic patterns for whole zein and for fractions of the same material precipitated from 49 and 45% aqueous alcohol. The patterns are almost identical. In order to eliminate the possibility that these fractions were contaminated with coprecipitated materials, zein was put through the multiple fractionation scheme shown in Fig. 7. The final fractions yielded similar electrophoretic patterns (Fig. 8).

In another test, a single fraction of zein was repeatedly dissolved in fresh 60% alcohol and precipitated by reducing the alcohol concentration to 52%. After twelve such precipitations, the material gave a pattern similar to those of Fig. 8. Fractionation from isopropyl alcohol and from ethylene glycol gave the same result, and fractional peptization of zein from corn was no better (Preparation 3). Fractionation from dilute (0.3 g./100 ml.) solution was not attempted but might yield interesting results.

Separation of the fast-moving portion of zein from the remainder was accomplished by withdrawing the desired portion from the Tiselius cell with a long needle and syringe. The fast-moving portion, being formed by dissociation of the slow-moving aggregate, should have an average molecular weight lower than the value for the aggregate. An osmotic pressure determination on the fast-moving material of fraction 11-A-35 gave a value of 6.02 cm. of alcoholic acetate buffer solution (density 0.8853) at a protein concentration of 0.475 g./100 ml. The whole of fraction 11-A-35 gave a pressure of 14.8 cm. at a concentration of 2.00 g./100 ml. in the same buffer.

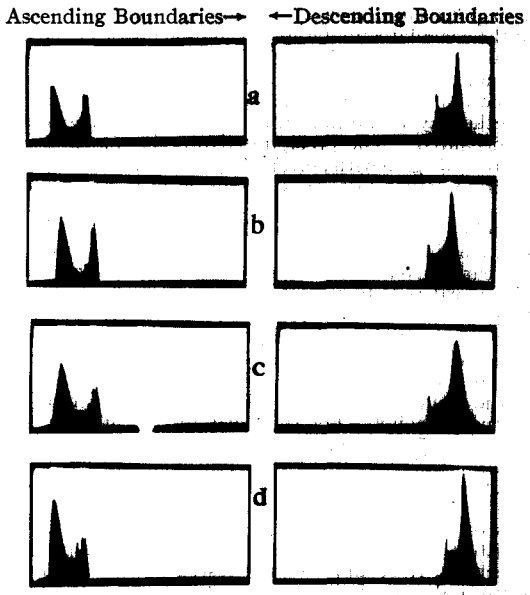

Fig. 8.—Electrophoretic patterns of samples from fractionation 1: a, fraction 1-C-55, 5.3 hours; b, fraction 1-C-50, 5.0 hours; c, fraction 1-C-45, 5.5 hours; d, fraction 1-C-35, 5.0 hours; all concentrations 1.2 g./100 ml. in 60% alcoholic acetate buffer, *p*H 5.4.

The average molecular weights corresponding to these values are 22,600 and 39,000, respectively. However, when a zein solution is diluted some material appears which can pass readily through a collodion membrane, and which consequently must have a molecular weight below 10,000.

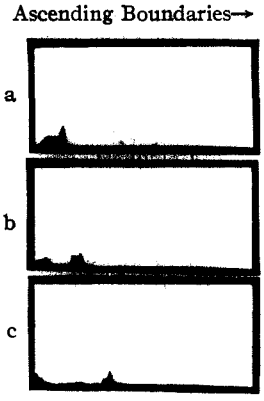

Fig. 9.—Electrophoretic patterns for the reworked fast-moving fraction of zein isolated in the Tiselius cell; concentration, 0.51 g./100 ml. in acetate buffer, *p*H 5.4, μ 0.016 in 60% alcohol; voltage 210, current 1.130 ma.; a, 5.5 hours; b, 11.3 hours; c, 20.5 hours. Descending boundary disrupted by bubbles.

If zein forms an association–dissociation system in the Sørensen sense, the fast-moving fraction isolated in the Tiselius cell should revert to a mixture of fast- and slow-moving components on reworking and concentration. Figure 9 gives the electrophoretic pattern of material so treated. A substantial portion of the fraction is now slow-moving. It differs from the slow-moving component from which it was derived in that it moves in the opposite direction at the same *p*H.

Experimental

Preparation of Zein.—For preparations 1 and 2, yellow corn (10–20 lb.) was ground through the 2 mm. screen of a large Wiley mill, then extracted with aqueous alcohol (72% by weight, 2–4 gal.) at room temperature for several days with occasional shaking. The extract was filtered, run slowly into about 3 volumes of cold 0.1 *M* potassium chloride solution with constant stirring, and the coagulated material was filtered off on a linen cloth. This material was redissolved in 72% alcohol, shaken with an equal volume of petroleum ether, and the mixture was run slowly into cold 0.1 *M* potassium chloride solution. The ether layer contained much of the oil and pigment and was decanted. The precipitated protein was redissolved and treated again with petroleum ether. The final product was dried over phosphorus pentoxide in a vacuum desiccator. All operations in which the protein was dissolved in alcohol were carried out at room temperature. All operations in which the protein was in contact with water were carried out at 6°. The dry product was brittle, easily powdered, light yellow in color. The yields were 8–24 g.

For preparation 3, ground yellow corn (22 lb.) was extracted for a week with alcohol (72%, 3 gal.), with occasional stirring every day. The extract was siphoned off and the residue extracted again for a week with a 3-liter portion of alcohol. For the third and all subsequent extractions, 1.5-liter portions of alcohol were used, with a contact period of one week and occasional stirring. Eleven extractions of the same corn were made, and each extract was purified in the manner previously described. The yields of the successive extractions were 7.0, 19.1, 16.2, 6.5, 7.6, 9.3, 5.6, 4.0, 5.0, 5.5 and 5.3 g., respectively. Extracts 1, 4, 7, and 10 were tested in the Tiselius apparatus and gave similar patterns.

Fractionation of Zein.—A preliminary test was made on preparation 2 by dissolving 2 g. of the material in 100 ml. of 72% alcohol,[12] adding enough water to make the alcohol concentration 55%, and allowing the solution to stand overnight at 6° in a centrifuge bottle. Precipitation was negligible, so the concentration was reduced to 49% and the solution was again allowed to stand overnight. By morning a layer of an oily, clear precipitate had appeared, and this was centrifuged off at 6°. The supernatant liquid was poured into another centrifuge bottle, and the same procedure was repeated at 45 and 35% alcohol concentration. The final 35% solution was discarded, since it contained only a trace of protein. The three precipitates were washed with several portions of absolute ether to remove the last traces of alcohol and water. Ether was removed in a stream of cold air. The yields were 1.391 g. from 49%, 0.498 g. from 45% and 0.046 g. from 35% alcohol.

For fractionation 1, 10 g. of commercial zein (Mazein, X grade[13]) was dissolved in 100 ml. of 70.85% alcohol to yield a hazy solution. Enough water (24.99 ml.) was added to make the alcohol concentration 55%, and 0.93 g. of potassium chloride was added to make the solution 0.1 *M* with respect to this salt. The solution was allowed to stand overnight at 6° and was then centrifuged free of the dark-colored suspended coagulum which had appeared. The sample was next fractionated as previously described, except that 50% alcohol concentration was used instead of 49%. Subsequent treatment of the fractions was carried out as indicated in Fig. 7.

After the first separation into four fractions, the portion precipitated from 55% alcohol was discarded, since it apparently contained mostly extraneous material and denatured protein. Each of the remaining three fractions was then separated in the same manner into four portions, giving twelve separate fractions. All of the portions precipitated from 55% alcohol were then mixed, and the portions precipitated at 50, 45 and 35% alcohol concentrations were recombined in the same manner, giving four twice-precipitated fractions. These were fractionated for the third time, yielding sixteen portions which were then recombined as before to give four thrice-precipitated fractions.

Fractionation 2 was carried out in exactly the same manner except that fractionation C was omitted. The original solvent in this case was 60% alcohol containing 0.5% sodium acetate, and a 0.5% solution of sodium acetate in water was added to reduce the alcohol concentration. All four twice-precipitated fractions gave electrophoretic patterns similar to those for fractionation 1.

In fractionation 7, commercial zein (1 g.) was dissolved in 100 g. of 60% aqueous isopropyl alcohol, buffered to *p*H 5.8 with acetic acid and sodium acetate (0.01 *M* with respect to sodium ion), and the alcohol concentration was reduced to 40%. A precipitate appeared overnight at 6° and was removed. The concentration of the supernatant liquid was reduced to 35%, at which point noncentrifugable material appeared.

In fractionation 9, 1 g. of commercial zein was dissolved in 100 g. of pure ethylene glycol, and the glycol concentration was reduced to 99% by addition of water. A haze formed which coagulated after two weeks and was then centrifuged off.

For fractionations 10 and 11, commercial zein (10 g.) was dissolved in 1 liter of acetate buffer (0.1 *M* with respect to total acetate) in 60% alcohol, *p*H 5.4, and the alcohol concentration was immediately reduced to 45%. After overnight standing at 6° the solution was filtered, and the filtrate was reduced to 35% alcohol concentration. After several days at 6°, the precipitate settled on the walls of the flask and the supernatant liquid was poured off. Fractionation 12 was similar, but additional precipitations were made at 55 and 50% alcohol concentrations.

Measurement of *p*H.—All *p*H values are the readings given by glass and saturated calomel electrodes in the Beckman Electrometer on buffer solutions only (zein absent), unless otherwise noted. Dole[14] has indicated that when the

(12) All alcohol concentrations are expressed on the weight basis.

(13) Courtesy of Corn Products Refining Co., Argo, Ill.

(14) Dole, THIS JOURNAL, **54**, 3100 (1932).

glass electrode is used in 60% alcohol solutions the error is only 5 millivolts or less than 0.1 *p*H unit.

Preparation of Buffers.—All buffers were made up to a definite ionic strength, so that the electrophoretic mobilities in different buffers could be compared directly. The effect of alcohol in all cases was to shift the *p*H about 1.5 units toward the alkaline side. Table I gives the compositions of the buffers used.

TABLE I

COMPOSITIONS OF BUFFERS

Buffer	NaOH, moles/liter	Acid, moles/liter	*p*H obs.	*p*H calcd.[a]
Acetate	0.016	0.100	5.4	5.4
Chloroacetate	.016	.100	3.6	3.4
Lactate	.016	.084	4.6	4.5
Succinate	.0112	.0065	8.2	8.4
Veronal	.016	.0192	10.0	9.9

[a] *p*H calculated from the Henderson equation, then 1.5 *p*H units added.

Critical Precipitation Curves.—Solutions of commercial zein (1 g. per 100 ml.) in 60% alcohol were prepared. These were buffered at various *p*H values with acetic acid–sodium acetate or lactic acid–sodium lactate mixtures of equal ionic strength, then titrated with water at room temperature until a definite precipitation had taken place, and the amounts of water required were noted (Fig. 5).

Electrophoresis Methods.—The apparatus used was made by the Klett Manufacturing Co. Zein samples to be tested were dissolved in about 15 ml. of buffer solution and then dialyzed overnight in a cellophane bag against the remainder of the buffer (about 1600 ml.). This dialysis was accomplished in the cold room at −5 to +5°. If any insoluble material appeared it was filtered off in the cold. The solution was then introduced into the 11-ml. Tiselius cell and electrophoresis was carried out in the usual manner. Most runs were made in 60% aqueous ethyl alcohol, in 0.1 *M* acetate buffer, *p*H 5.4, ionic strength 0.016; the temperature was 1 to 3°, the applied voltage 315, the current 1.5 to 15 milliamperes (potential drop about 9.6 volts/cm.). Any variations from these conditions are noted under the appropriate patterns in the figures. Photographs of the boundaries were made at about two, five, ten and twenty-four hours from the start of the experiment, with the Philpot lens in place.

In the experiments in which the Tiselius apparatus was used to fractionate zein solutions, the components were separated by electrophoresis, the current was stopped, the center section of the cell was moved to the right, and the buffer solution above the center section was removed. The cell was then realigned and a six-inch needle was used with a syringe to remove the portions of solution desired. The position of the needle in the solution was observed on the ground glass of the camera.

Measurement of Osmotic Pressure.—In preliminary experiments on fraction 11-A-35 with the Fuoss–Mead[15] osmometer, collodion membranes proved to be somewhat permeable to a part of the protein. Successive runs on the same solution gave rapidly decreasing osmotic pressure readings. The decrease in pressure was more rapid than the increase in concentration of protein on the buffer side of the membrane (judged by turbidity on adding a drop to water) showing that the material diffusing through the membrane was of low molecular weight.

Even the rising and falling curves on the same solution, determined about thirty minutes apart, showed a difference in indicated pressure, and trailed off in the direction of lower pressure at about the same rate. These curves were extrapolated to zero time to determine the pressure at the beginning of the run. Such extrapolation is obviously inaccurate, but gives a fair estimate of the osmotic pressure.

To eliminate uncertainty caused by porosity of collodion, the osmotic pressure of the fast-moving fraction isolated in the Tiselius apparatus was determined with a cellophane membrane (DuPont 600 PT), which turned out to be very slow but impermeable to protein. The Fuoss–Mead osmometer, having a closed side, was subject to temperature variations, so a modified Urban osmometer[16] was used. Equilibrium was attained after a period of two weeks at room temperature. Acetate buffer, *p*H 5.4 in 60% alcohol, was used in all osmotic pressure work.

Discussion

The main contribution of Tiselius to electrophoresis technique was reduction of the tendency toward boundary disturbances caused by heating. He accomplished this by using flat, thin cells and by using a temperature just below the temperature of maximum density of water. Since 60% alcohol has no point of maximum density (at least in the readily accessible temperature range), heating effects in this solvent are somewhat greater than in water. The amount of boundary disturbances depends on the power loss (I^2R), the coefficient of expansion of the solution, and the heat capacity of the solution. In comparing the disturbance effect in 60% alcohol with that in water, it is found that the power loss in alcohol is considerably less at a given voltage; the high resistance of the solution permits passage of only a small current, which is the more important factor in the power loss. The coefficient of expansion of alcohol, however, is much greater than that of water at 2°, and its heat capacity is somewhat lower. These factors tend to make the disturbance effect greater in alcohol. The tendency toward boundary disturbances in the experiments reported here is about five times as

(15) Fuoss and Mead, *J. Phys. Chem.*, **47**, 59 (1943).

(16) Urban, *Rev. Sci. Instruments*, **5**, 375 (1934).

great as it is in the usual experiments in aqueous solution at 2°, but half as great as in aqueous solution at 25°.

Although the fast-moving peaks in the zein patterns are sharper than the peaks obtained from water solutions of proteins, there are several indications that they represent actual zein components and are not false peaks caused by convection currents. Many successful experiments have been carried out in aqueous solution at 25° under heating conditions worse than these. Visual observations of the zein boundaries were made in each case and revealed no abnormalities except slight curvatures at the interfaces after long-continued electrophoresis. Changes in *p*H of the medium while all other factors were held constant produced marked changes in the patterns (Fig. 3), although the heating effects were very nearly the same. Strong evidence is given by Fig. 2d and 3a, which represent the extreme heating cases. The patterns in Fig. 2d were obtained under conditions such that the heating effect was eight times that in the standard 0.1 *M* buffer, and show practically no tendency toward separation into peaks. Figure 3a was obtained with only half the usual heating effect and shows a definite separation into peaks.

The difference between the ascending and descending zein boundary patterns is explained by the effect of concentration on zein dissociation. At the ascending boundary, the fast-moving components move into the buffer and are continuously replaced in the main body of the solution by similar complexes moving at the same rate. Thus there is no disturbance of equilibrium in the main body of solution. If the original concentration is 1 g./100 ml., the concentration of fast-moving components at the ascending boundary is now about 0.3 g./100 ml. at which concentration zein has very little tendency to associate. The concentrations of components in the main body of solution have not altered, but at the descending boundary the slow-moving component now has a concentration of about 0.7 g./100 ml., at which concentration it tends to dissociate to form more of the fast-moving components. Since dissociation is relatively slow (requiring approximately two hours for equilibrium in the original fractionation) the fast-moving components should be formed slowly and continuously. The pattern should then show a large peak for the aggregate and a continuous slope indicating gradual formation of fast-moving components. This is observed. The small leading peak is formed by the fast-moving material present at the beginning of electrophoresis.

The extensive data of Watson and Williams[6,7] prove that when zein is fractionated by addition of water to its alcoholic solution the fractions have different average molecular weights. These authors reject the Sørensen association–dissociation idea for zein principally because its fractions are stable and do not revert to the original condition on long standing. This is undoubtedly a fact, but it can also be explained by the dissociation hypothesis. The individual zein components attain different equilibria at the various alcohol concentrations used in fractionation, and the precipitated fractions vary in their proportions of the basic components. On solution in the same solvent they attain different equilibria, but each fraction is stable.

The present experiments indicate that each of the fractions consists of an association–dissociation system. The slow-moving components revealed by electrophoresis to be present in the various fractions must therefore be in different states of aggregation (contain different numbers and proportions of the individual components) and have different molecular weights, although their surface charges are so similar that their mobilities are very nearly the same. In one case the mobility of the slow-moving component is noticeably different. Fractionation in the Tiselius cell followed by reworking of the fast-moving components results in partial association to an aggregate (Fig. 9) which moves slowly in a direction opposite to that of the slow-moving components of water-precipitated fractions. The fast-moving components (the dissociation products) are of course the same for all fractions.

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Summary

1. Zein solutions contain at least six components of different electrophoretic mobility, which constitute reversible association–dissociation systems.

2. The equilibrium between components is easily disturbed by changes in protein concentration, buffer concentration, or *p*H of the solution.

3. It is not possible to separate the components by fractional precipitation from solutions of ordinary concentration, but they can be separated in the Tiselius electrophoresis apparatus.

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