ture differ little in stability. This absence of detectable moment when a phenyl and a vinyl group are attached to each other is consistent with the fact that the attachment of a methyl group to a vinyl to form propylene gives practically the same moment as the attachment of a methyl group to phenyl to form toluene. It should be recalled, however, that the attachment of a group producing large charge displacement, such as cyanide or chlorine, to the phenyl or the vinyl group does not give the same moment in the two cases.

Summary

The dielectric constants of the vapors of 1,2-dimethyl-, 1,3-dimethyl-, and 2-ethylbutadiene, and of cyclopentadiene and styrene have been measured and used to calculate the dipole moments of the molecules. The moment values are consistent with the molecular structures required by the previously discussed theory of hyperconjugation and resonance, thus lending support to the theory.

PRINCETON, N. J. RECEIVED NOVEMBER 14, 1945

[CONTRIBUTION FROM THE DEPARTMENT OF BIOLOGY, MASSACHUSETTS INSTITUTE OF TECHNOLOGY]

A Fibrous Modification of Insulin. I. The Heat Precipitate of Insulin

BY DAVID F. WAUGH

When insulin is heated in the presence of dilute acid a series of changes take place, the end result of which may be the formation of a visible flocculent precipitate. This flocculation, termed the "heat precipitate" by du Vigneaud, forms most rapidly in the presence of sulfuric acid and salt.[1,2,3,4]

Slightly different conditions lead to the formation of a clear gel which shows either flow or static double refraction depending on the protein concentration. The insulin has been modified to give anisodiametric micelles.[5] These have been shown to be fibrils.[6]

The author will discuss aspects of fibril formation (such as reversibility, reactions involved, reaction kinetics and chemical modification of fibril formation) in other publications of this series. To interpret the results of such studies an understanding of the relationships between fibril and floccule formation is necessary. Fibril formation seems to be a prerequisite for floccule formation. However, floccule formation does not always follow fibril formation, for preparations may be obtained which contain only fibrils or varying proportions of fibrils and floccules.

This publication considers the structure of the floccule and correlations between rates of fibril and floccule formation. As will be shown the floccule has a spherocrystalline structure. A number of intra- and extra-cellular structures have a similar arrangement of their component parts. Insulin spherites are therefore of considerable interest.

(1) Blatherwick, Bischoff, Maxwell, Berger and Sahyun, *J. Biol. Chem.*, **72**, 57 (1927).
(2) du Vigneaud, Geiling and Eddy, *J. Pharmacol.*, **33**, 497 (1928).
(3) Gerlough and Bates, *ibid.*, **45**, 19 (1932).
(4) du Vigneaud, Sifferd and Sealock, *J. Biol. Chem.*, **102**, 521 (1933).
(5) Langmuir and Waugh, THIS JOURNAL, **62**, 2771 (1940).
(6) Waugh, *Am. J. Physiol.*, **133**, 484 (1941).

I. Experimental Techniques

The insulin[7] solutions to be heated were sealed into annealed Pyrex ampules made from 8-mm. tubing. Each ampule, 20 cm. long, had a total volume of about 3 ml. One ml. of solution was generally enclosed.

Double refraction measurements to be reported here were usually made in the presence of varying quantities of floccules. Quantitative measurements were therefore not attempted. Rocking the tube between crossed polaroids produced sufficient flow so that the appearance of faint or strong flow or weak, strong, or intense static double refraction could be timed conveniently. Only the longer fibrils, estimated to be 5–10 thousand ångström units long (assuming an axial ratio of 50–100), are oriented by this means if flow double refraction is obtained. Static double refraction, which depends on hindrance of free rotation, may also involve much shorter fibrils.

Microscopic examinations were made with a standard petrographic microscope. Information concerning analysis by polarized light, double refraction, and particularly the properties of objects having radial symmetry may be found in recent reviews.[8]

Crystalline insulin was used except where noted.

II. Insulin Fibrils

A few results are presented as a preparation for discussing floccule formation. When heated at 100° a sample of 2% amorphous or crystalline insulin in hydrochloric or phosphoric acids at pH 2.3–2.5 generally shows little or no floccula-

(7) The author is indebted to Dr. G. H. A. Clowes of Eli Lilly & Co. for his generous gifts of amorphous insulin and to Dr. H. Sydney Newcomer for making available crystalline insulin prepared by Squibb and Sons.
(8) Schmitt in "Medical Physics," edited by Glaser, New York Book Publishers, New York, N. Y., 1944.

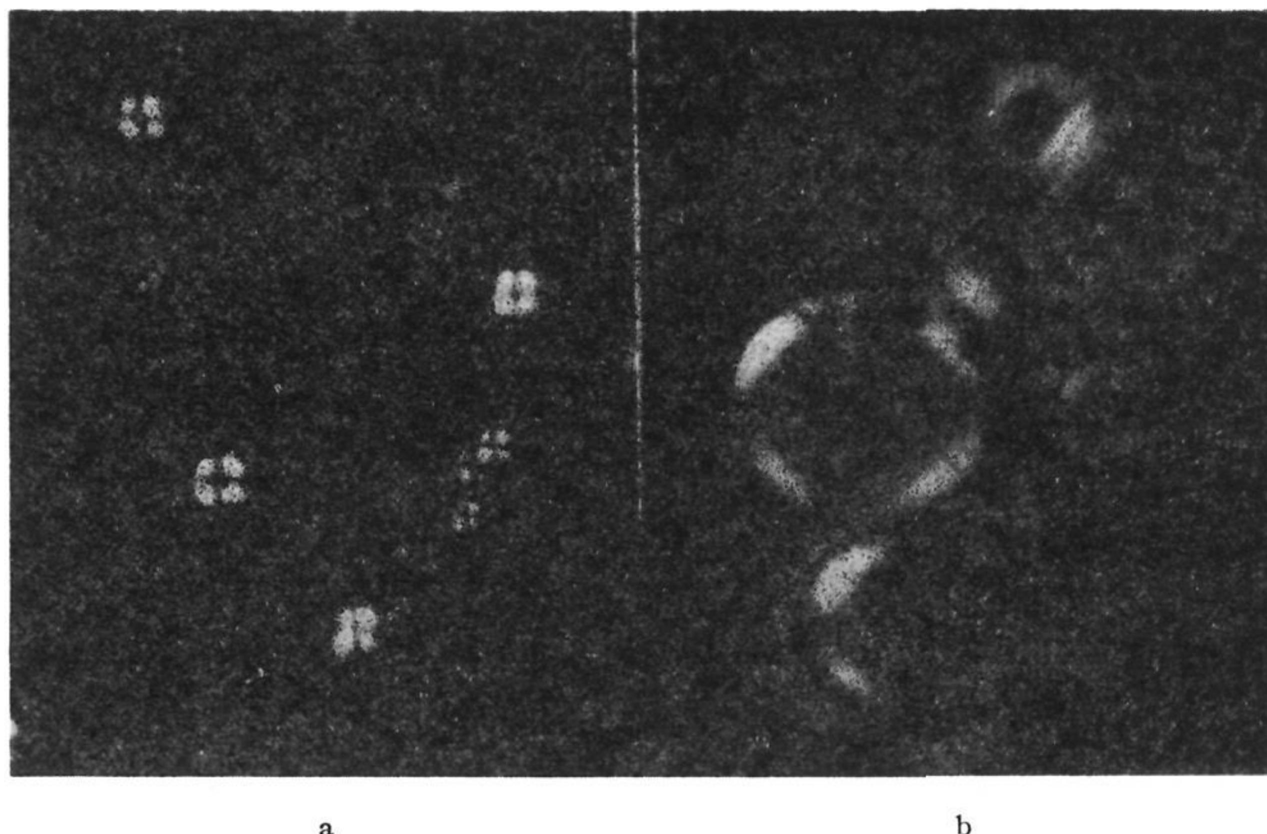

Fig. 1.—Photomicrographs of spherites taken with crossed nicols. The contrast of b has been increased photographically; 100 microns equals 30 mm. (a) Small compact spherites produced in 2% insulin and 1% sodium chloride in hydrochloric acid at *p*H 1.8 after 3 min. at 100°. (b) Loose spherites produced in 0.5% insulin in hydrochloric acid at *p*H 1.0 after 63 min. at 100°.

tion but remains clear except for the faint light scattering indicating asymmetric particles. The solution forms a thixotropic gel which, in the gelled state, acquires static double refraction after an initial disturbance. Quantitative measurements of flow double refraction obtained after diluting the original gel show that the majority of the insulin has been transformed into the fibrous form.

Recently, C. E. Hall of this department has obtained direct information by taking electron micrographs of insulin fibrils prepared in hydrochloric acid. These fibrils have lengths of about 16,000 Å. and widths varying between 100 and 180 Å. In general the widths are quite uniform, averaging 140 Å. If the figures given are diameters of rods the extremes in cross section area have a ratio of 3.2.

III. The Structure of the Insulin Floccule

Insulin may be quantitatively converted into the floccule form by heating at 100° in 0.1 *N* hydrochloric or sulfuric acids.[2] The presence of neutral salt greatly speeds up the process.

Floccules prepared in a variety of ways have been examined with a petrographic microscope. Although varying in size and compactness, all undeformed floccules possess a polarization cross the dark arms of the cross being parallel to the distinguishing directions of the nicol prisms (Fig. 1). Introduction of a Red I plate shows the cross to be positive in character. The larger floccules are made up of smaller ones. They do not show crosses themselves but portions of the crosses of their component parts may be seen. The floccules, then, are spherocrystals whose sub-units are disposed either radially or tangentially. If insulin fibrils make up the spherites the fibrils must be oriented radially for the following reasons: Suspending fibrils in media of different refractive indices and drying a film of oriented fibrils on a glass slide shows that the double refraction is almost entirely from double refraction. In order to obtain the positive polarization cross described above fibrils must have a statistical radial orientation.

That spherites are composed of fibrils was shown by dispersing the spherites in dilute alkali. A temperature of 0° was chosen. At higher temperatures the spherites not only disperse into fibrils but the fibrils disrupt further. Little happens to spherites below a *p*H corresponding to a room temperautre *p*H of about 11.0. Between *p*H's of 11.0–11.5 dispersal takes place for the clumps of spherites and the spherites themselves disappear with time. The solution, which may have no detectable flow double refraction originally, now exhibits increasing amounts. Flow double refraction builds up to a persistent maximum showing that the particles into which the spherites have dispersed are stable under these conditions. The double refraction has the same sign and approximately the same magnitude as that of a suspension of fibrils of the same concentration. This is considered sufficient evidence that the spherites are constructed of fibrils.

At *p*H values above 12.0 spherite dispersal takes place rapidly. At the same time flow double re-

fraction builds up to a maximum and then declines, more rapidly the higher the *p*H, indicating that the fibrils are now disrupting. This is in agreement with the disruptive effects of alkali on suspensions of fibrils.

In the presence of salt a considerably higher *p*H is necessary for dispersal. With high salt concentration (1% sodium chloride) disruption of fibrils may keep pace with spherite dispersal.

IV. Factors Affecting Spherite Formation

The formation and compactness of spherites depends upon variables including *p*H, protein concentration, the anion of the acid, presence of salt, temperature and viscosity.

(1) ***p*H.**—The rate of flocculation increases with decreasing *p*H. Down to *p*H's of about 3.0 both fibril and spherite formation are lacking. At 100° 2% insulin in hydrochloric acid shows the first signs of flocculation after ten to fifteen minutes at *p*H 2.0 and one to two minutes at *p*H 1.1.

(2) **Protein Concentration.**—At 100° and *p*H 1.0 2% insulin starts flocculating in one to two minutes while 0.5% takes twenty-five minutes.

(3) **Anion of Acid.**—1.88% insulin at *p*H 1.85 (±0.05) dissolved in sulfuric, hydrochloric and phosphoric acids shows the following at 100°: The sulfuric acid sample became opaque in four minutes, the hydrochloric acid sample showed fair numbers of large floccules after thirty-seven minutes while the phosphoric acid sample showed no tendency to flocculate. These results are in agreement with the studies of others on the heat precipitate.[3,4] Other ampules were examined for flow double refraction. The sulfuric acid ampule showed, if anything, the faintest flow double refraction. After thirty-seven minutes the hydrochloric acid ampule contained a thin gel which showed faint static double refraction as a background to numerous floccules. After forty-five minutes the phosphoric acid ampule contained a strong clear gel which showed intense static double refraction.

(4) **Temperature.**—As has been demonstrated by others[3,4] flocculation has a Q_{10} of about 4.0 in sulfuric acid.

(5) **Effect of Inorganic Salt.**—Using a *p*H of 2.0 obtained with phosphoric acid and in the presence of 0.04 *N* sodium sulfate, chloride or phosphate 2% insulin shows almost complete flocculation in two to four minutes at 100°. The most effective agent is given first.

Part of the effect of salt on flocculation is probably due to the expected decrease in the repulsive forces between similarly charged particles when the ionic strength is increased. Neutral salts, however, greatly increase the rate at which fibrils form. This factor will be considered shortly.

(6) **Viscosity.**—The addition of glycerol, 10–20% by volume, will suppress flocculation in those cases where the tendency to flocculate is not pronounced (2% insulin in hydrochloric acid at *p*H 1.8–2.0, temperature 100°). At the same time fibril formation proceeds normally.

V. The Nature of Spherite Formation

The structure of the spherite is one of statistical radial orientation of the constituent fibrils (see III). A new fibril entering the association must diffuse into the region of the growing spherite and become properly oriented. For reasons involving steric hindrance one would expect the shorter fibrils to be recruited and oriented most easily. The elongation of those fibrils already present would constitute a second means of spherite growth.

In a subsequent publication data will be presented which show: (a) the rate of fibril formation increases with increasing hydrogen ion concentration, salt concentration, protein concentration and temperature. (b) Two main reactions are involved in fibril formation. The first, which is generally the slower of the two, is the formation of active centers, the second being the elongation of these centers into extended fibrils. The relative rates of these reactions determines the balance between fibril concentration and average fibril length.

Conditions may be chosen from section III so that solutions of 2% insulin at 100° show spherite (floccule) formation (a) not at all or only after prolonged heating (phosphoric acid), (b) after some ten to twenty minutes (hydrochloric acid, *p*H 2.0–2.3), (c) two to three minutes after start of heating (hydrochloric acid, *p*H 1.1) and (d) within one minute (hydrochloric acid–sodium sulfate or chloride, *p*H 1.5–2.0).

In those cases where spherites do not form (see (a) above) the double refraction builds up to faint static values during the first few minutes and then increases slowly until intense static values are reached. The solution gels during the process. Under these conditions active center formation is slow. The fibrils, on the average, are too long to aggregate into typical spherites. A loose tactoid-like type of association is evident for clear needle shaped masses of linearly oriented fibrils may be detected by small differences in light scattering and by their double refraction. In distinction to spherites such associations may be dispersed by dilution with acid or distilled water.

Where spherites appear after ten to twenty minutes (see (b)) flow double refraction having a relaxation time of the order of a second makes its appearance first. The spherites, which then appear, remove fibrils about as fast as they are formed for the double refraction does not go beyond weak static values. Figure 1b shows large poorly oriented spherites. The loose structure, attributed to the fact that longer fibrils are forming spherites, is shown by the hazy nature of the polarization cross.

Where spherites form within a minute or so (see (c) and (d)) double refraction, when detectable under the conditions used, has a very short time (less than 0.01 second) indicating a fair concentration of short fibrils. This favors spherite formation for, as shown in Fig. 1a, the spherites resulting from this type of treatment are small, compact, and show well defined polarization crosses.

For purposes of comparison fibrils having lengths of 10, 20, 40 and 100 thousand ångström units have relaxation times of 0.03, 0.2, 1.39 and 18.8 seconds. These values were calculated from equation 13, page 511 of Cohn and Edsall,[9] assuming $\beta = 70 \times 10^{-8}$ cm., $\eta = 0.01$ poise and $T = 300°$.

(9) Cohn and Edsall, "Proteins, Amino Acids and Peptides," Reinhold Publishing Corp., New York, N. Y., 1943.

Summary

In acid solution insulin may be modified to form highly asymmetric fibrils. The aggregation of fibrils into spherites in which the fibrils are radially oriented accounts for the visible heat precipitate of insulin. The rate of spherite formation increases with increasing hydrogen ion concentration, protein concentration, neutral salt concentration, temperature and fluidity. In the absence of salts the acid anion has a pronounced effect on fibril and spherite formation.

Fibril formation precedes spherite formation. Spherite formation, favored by a high concentration of short fibrils, is absent under those conditions which lead, initially, to low concentrations of very long fibrils.

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[CONTRIBUTION FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY, No. 1021]

The Reactions of Antiserum Homologous to the *p*-Azophenyltrimethylammonium Group[1]

BY DAVID PRESSMAN, ALLAN L. GROSSBERG, LELAND H. PENCE, AND LINUS PAULING

A great amount of information about the nature of serological reactions has been obtained through experiments on the properties of antisera produced by animals on injection of artificially conjugated proteins, especially azoproteins. This work, carried out during the past quarter of a century by Landsteiner and his collaborators[2] and by other investigators, has dealt mainly with the reactions of antisera with azoproteins and simple substances containing negatively charged haptenic groups (azophenylarsenate, azobenzoate, etc.) or neutral groups (azophenyl, etc.). The only serological study of positively charged haptenic groups which has been reported is that of Haurowitz and his collaborators,[3] who prepared antiserum by injecting rabbits with an azoprotein containing the *m*-azophenyltrimethylammonium group, which was made by the reaction of sheep serum globulin with diazotized trimethyl-(*m*-aminophenyl)-ammonium ion; this antiserum was found to precipitate the immunizing azoprotein and also similar azoproteins made from bovine serum globulin and ovalbumin, and the precipitation was found to be inhibited by a simple dihaptenic substance, di-(*m*-azophenyltrimethylammonium)-tyrosine.

(1) The Serological Properties of Simple Substances. XII. For no. XI of this series see D. Pressman, A. B. Pardee, and L. Pauling, THIS JOURNAL, **67**, 1602 (1945).

(2) K. Landsteiner and L. Lampl, *Biochem. Z.*, **86**, 343 (1918); K. Landsteiner, "The Specificity of Serological Reactions," Charles C. Thomas, Springfield, Ill., 1936.

(3) F. Haurowitz, K. Sarafyan, M. M. Yenson, S. Berkol, and P. Schwerin, *Rev. Fac. Sci. Univ. d'Istanbul*, **A5**, 1 (1940); F. Haurowitz, K. Sarafyan, and P. Schwerin, *J. Immunol.*, **40**, 391 (1941); F. Haurowitz, *ibid.*, **43**, 331 (1942).

Extending our studies of the serological properties of simple substances, we have now prepared an antiserum homologous to a positively charged haptenic group, the *p*-azophenyltrimethylammonium group, and have studied its reactions with a large number of substances. The antiserum used (called anti-A_p serum in the rest of this paper) was made by injecting rabbits with sheep serum coupled with diazotized trimethyl-(*p*-aminophenyl)-ammonium chloride. Studies were made of the precipitation of this antiserum by two azoproteins containing the same haptenic group, A_p-ovalbumin and A_p-horse serum albumin, of the inhibition of precipitation in these systems by a score of haptens, and of the effect of change of hydrogen-ion concentration on these reactions.

Experimental Methods

Protein Antigens.—The immunizing antigen used for inoculating the rabbits was made by diazotizing three portions of trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride weighing 0.10, 0.24, and 0.43 g., respectively, coupling these at *p*H 8.0 to 8.5 and 5° with three 67-ml. portions of sheep serum, and finally mixing the three preparations, on the assumption that such a mixture would cover the range of highest antigenicity. When the mixture was brought to *p*H 4.6 only a slight amount of precipitate formed. The *p*H was brought to 7 and the solution was dialyzed against saline solution.

Test antigens were made from crystallized hen ovalbumin and from crystallized horse serum albumin by reaction with diazotized trimethyl-(*p*-aminophenyl)-ammonium chloride hydrochloride. Preparations 1 and 2 of A_p-ovalbumin were made by coupling 0.1-g. and 0.45-g. portions of the diazotized amine at *p*H 9 with 0.8 and 5.0 g. of ovalbumin, respectively. The antigens were purified by precipitating twice at *p*H 4.9, redissolving each time at