[CONTRIBUTION FROM THE COATES CHEMICAL LABORATORIES, LOUISIANA STATE UNIVERSITY]

The Ovalbumin–Chloroauric Acid Reaction

BY J. P. CRAIG, JR., A. G. GARRETT AND H. B. WILLIAMS

RECEIVED SEPTEMBER 28, 1953

The reaction between ovalbumin and chloroauric acid was studied in buffered solution acid to the isoelectric point of the protein, by means of a photoelectric colorimeter and a light scattering photometer. The reaction was investigated under different conditions of *p*H, temperature and concentration of reactants. It was characterized by rapid reaction of the protein with chloroaurate ion followed by aggregation of the protein–chloroaurate complex and subsequent flocculation of the aggregates. The data suggest that the aggregation occurred in two stages, *i.e.*, the formation of primary aggregates which through a preferred direction of growth, or through rearrangement, changed over to aggregates of lower dissymmetry prior to flocculation. The effects of the different experimental conditions on the "induction period," the limiting optical density and the rate of flocculation were summarized and explained where adequate data were available. The data support a reaction mechanism between ovalbumin and chloroaurate ion whereby the latter combines with the $-NH_3^+$ groups of the protein, HCl is liberated and coördination results between the nitrogen atom of the amino group and the gold atom of the $AuCl_3$. On the basis of the number of hydrogen ions liberated during the course of the reaction it was calculated that the number of chloroaurate ions reaction per molecule of ovalbumin was equal to the number of basic groups of the protein. The possibility of using chloroauric acid as a reagent for determination of the basic amino groups of a protein is suggested.

Introduction

The combination of proteins with heavy metal ions has been the subject of many investigations. The initial experiments were those of Loeb,[1] who as a consequence of his observations suggested that a protein below its isoelectric point would probably combine with anions and not cations, while the reverse would be true above the isoelectric point. In general two types of investigations have been conducted: (a) those studying the conditions of protein–ion combination as related to the stoichiometry of a given system[1–5]; and (b) those specifically investigating the observable physical processes of denaturation and coagulation.[6–10]

The investigation reported here concerns the effects of *p*H, concentration and temperature on the reaction between ovalbumin and chloroauric acid in media acid to the isoelectric point of the protein. The reaction, which produced a white coagulum, was followed initially with a photoelectric colorimeter. Evidence of a pronounced induction period, the duration of which was affected by all the aforementioned conditions, led to further investigation with a light scattering photometer.

Experimental Procedures

Photoelectric Colorimeter Experiments.—In a typical colorimeter experiment 10 ml. of a chloroauric acid solution of the desired concentration was added to 10 ml. of the appropriate ovalbumin solution, or *vice versa*, in the colorimeter tube. The tube was placed in an Evelyn photoelectric colorimeter and the transmittancy at a wave length of 620 mμ was recorded at half-minute intervals.

Absorption curves for ovalbumin and chloroauric acid were separately determined by use of a Beckman spectrophotometer. It was found, in agreement with other workers,[11] that no correction for protein absorption was necessary when light of wave length 620 mμ was used. When computing the optical density, corrections were made for (a) reacting chloroauric acid solutions—not more than 0.5% transmission; (b) non-uniformity of the colorimeter tubes; and (c) the non-linearity of the colorimeter galvanometer.

After the optical density reached its final value (Fig. 1) the reaction product was separated from the mixture by centrifugation and decantation, and the supernatant liquid was qualitatively analyzed for protein. The biuret, xanthoproteic, sulfosalicylic acid and glyoxylic acid tests for protein were used and gave negative results in all cases. The sensitivities of these tests, in particular the biuret and sulfosalicylic acid tests, are such that any significant amount of unreacted protein would have been detected. Control protein in the absence of chloroauric acid gave a positive test in all instances. From these observations it was concluded that the protein was entirely reacted in the presence of excess chloroauric acid. Various colorimetric methods were employed to determine the amount of chloroauric acid left in the reaction mixture; consistent results were not obtained by any method at low concentration levels of the chloroauric acid encountered in these experiments.

The chloroauric acid solutions and the protein solutions were buffered to the desired *p*H and were all of ionic strength 0.02. The buffered solutions had sufficient capacity to keep the *p*H constant when the chloroauric acid was added. In unbuffered solutions the *p*H was observed to decrease during the course of a reaction.

The temperatures at which the runs were made were those available in certain constant temperature rooms.

Light Scattering Experiments.—The Brice–Speiser light scattering photometer manufactured by the Phoenix Instrument Company was used for these studies.

A number of experiments were conducted in search of a ratio of chloroauric acid–ovalbumin concentration which would produce an induction period of convenient duration and yet have a protein concentration suitable for light scattering studies. On the basis of these preliminary experiments, 20 ml. of a 0.5% ovalbumin solution and 20 ml. of a 0.00382 *M* chloroauric acid solution were allowed to run together from pipets into the light scattering cell. Both solutions were of ionic strength 0.2 and buffered at *p*H 3.33.

During the course of several experiments the following quantities were measured as a function of time, with unpolarized incident light used in all instances: (1) the intensity of the transmitted light at 547 mμ, (U_u^{547}), (2) the intensity of the transmitted light at 405 mμ, (U_u^{405}), (3) the horizontal (H_u) and vertical (V_u) components of the light scattered at 90° to the incident beam at 547 mμ, and (4) the intensities of the scattered light at 45° ($I_{45°}$) and 135° ($I_{135°}$) to the incident beam at 547 mμ.

Before making the dissymmetry measurements (I_{45}/I_{135}) in which dust particles are extremely detrimental, the buffered chloroauric acid solutions and the buffer in which the protein was later dissolved were passed through an ultrafine sintered glass filter. For these experiments also, the reaction cell was inverted over the end of a glass tube delivering acetone vapor which condensed on the inner walls of the cell and streamed down the walls carrying virtually all of the dust particles with it. The buffered ovalbumin solutions were centrifuged for one hour at 15,000 r.p.m. before transfer to the light scattering cell. These transfers were always made with pipets which had been cleaned in hot

(1) J. Loeb, *J. Gen. Physiol.*, **1**, 39 (1918).
(2) E. Heymann and F. Oppenheimer, *Biochem. Z.*, **199**, 468 (1928).
(3) K. Kodama, *J. Biochem. (Japan)*, **2**, 505 (1923).
(4) J. N. Mukerjee and N. A. Sen, *J. Chem. Soc.*, **115**, 461 (1919).
(5) S. Oden, *Kolloid Z.*, **26**, 160 (1920).
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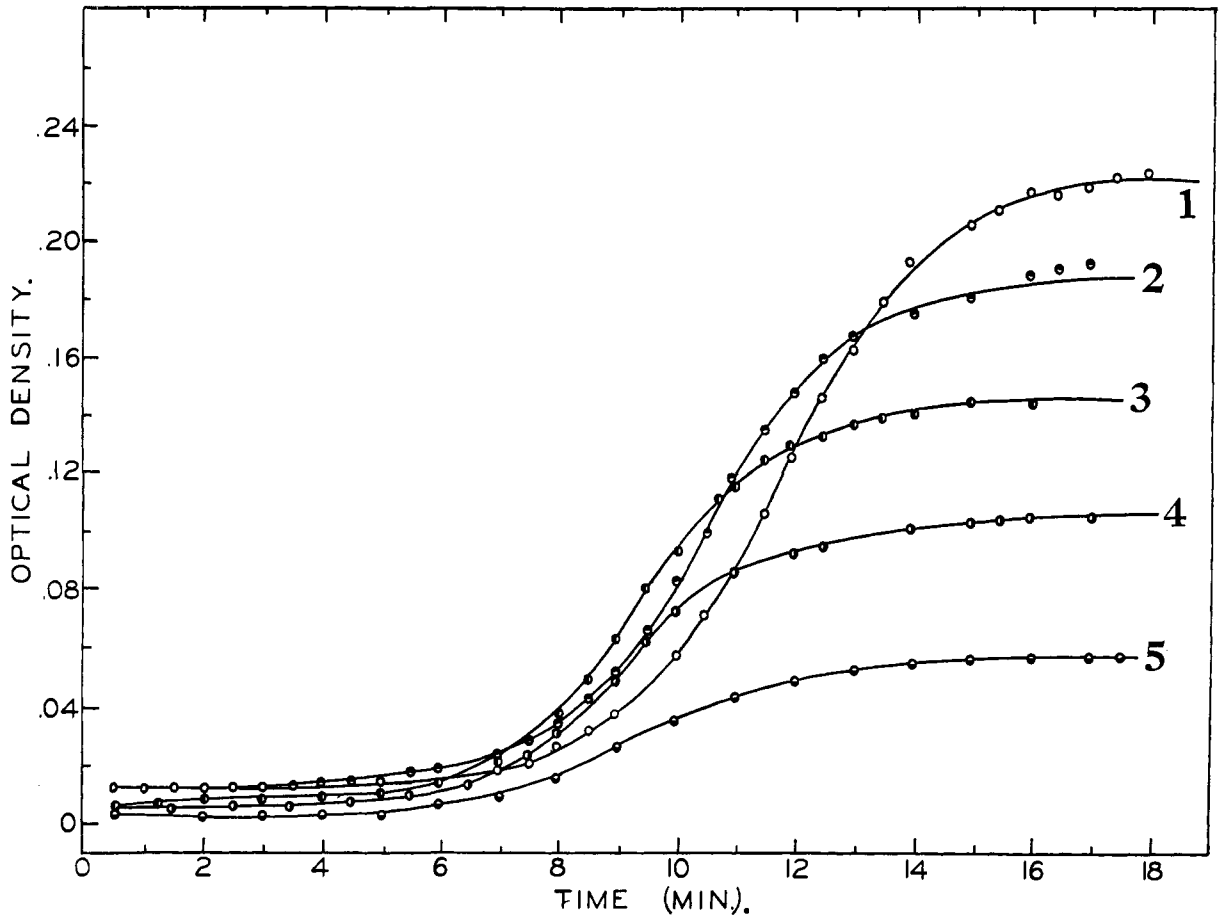


Fig. 1.—Optical density *vs.* time (*p*H 3.80, Au concn. 0.00183 *M*, temp. 22.5°; ionic strength 0.02): (1) ○, protein concn. 71 mg./100 ml.; (2) ◓, 57 mg./100 ml.; (3) ◐, 43 mg./100 ml.; (4) ◑, 29 mg./100 ml.; (5) ◒, 14 mg./100 ml.

chromic acid, rinsed with water of light scattering quality, and then rinsed once with a portion of the solution to be transferred. These precautions kept the number of dust particles and the amount of denatured protein to a minimum.

Preparation of Materials.—The ovalbumin was prepared by the method of La Rosa.[12] The thrice recrystallized protein was dialyzed against distilled water until an aliquot of the dialysate failed to give a positive test for sulfate ion upon the addition of crystals of barium nitrate. The dialyzed ovalbumin solution was then frozen and the water removed by vacuum sublimation. Concentrations of solutions made from the ovalbumin were determined by one of two methods. In the first method, the dry weight was determined after evaporation of aliquots of the protein stock solution at a temperature of 75–80°. The second method of analysis was the turbidimetric method of Exton[13] which is accurate to within 2.0%.

The chloroauric acid solutions used in this study were obtained by dilution of a concentrated stock solution of $AuCl_3 \cdot H_2O \cdot HCl$ which had been standardized by conventional gravimetric procedure.

Sodium acetate–acetic acid buffers of desired *p*H and ionic strength were employed.

Treatment of Experimental Data and Discussion of Results

Photoelectric Colorimeter Experiments.—Figure 1 shows typical plots of optical density *vs.* time for the ovalbumin chloroauric acid reaction. These curves bear a strong resemblance to those in autocatalytic or consecutive reaction processes.

(12) W. La Rosa, *Chemist Analyst*, **16**, 3 (1927).

(13) W. G. Exton, *J. Lab. Clin. Med.*, **10**, 722 (1925).

Data pertinent to the induction period are presented in Table I. The "induction period" was defined as that time corresponding to the intersection of extensions of the linear sections of the initial and middle portions of the "S" shaped optical density–time curves. Toward the end of the induction period it became apparent that a second process, observably one of flocculation, began to occur. There was an interval of time during

TABLE I

DEPENDENCE OF INDUCTION PERIOD ON *p*H, TEMPERATURE AND $AuCl_4^-$ CONCENTRATION

*p*H	Molar concn. of $AuCl_4^-$	Length in induction period in minutes Temperature[a] 6.5°	20.5°	31.0°
3.47	0.00125	78.0	22.5	8.3
	.00163	30.6	8.5	3.3
	.00250	2.6	1.1	<1.0
	.00375	<.5	<.5	<.5
3.88	0.00125	35.5	11.3	4.0
	.00163	15.8	4.8	1.8
	.00250	<1.0	<1.0	<.5
	.00375	<.5	<.2	<.2
4.33	0.00125	10.4	3.3	1.0
	.00163	3.5	<1.0	<.5
	.00250	<.2	<.2	<.2
	.00375	<.1	<.1	<.1

[a] Temperature control, ±0.5°.

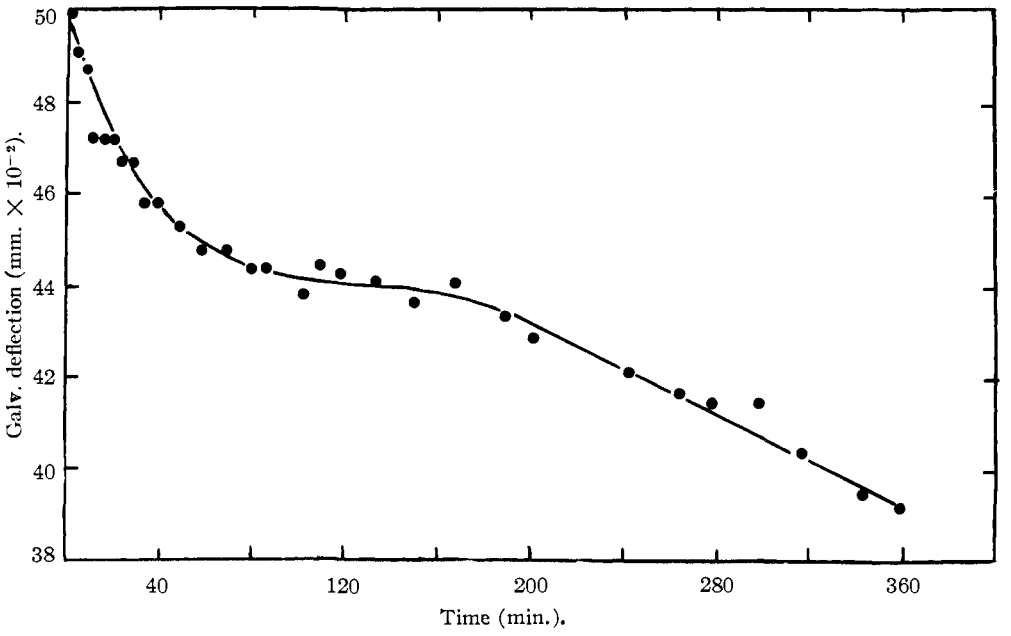


Fig. 2.—Intensity of transmitted light $U\mu_0$ (λ 547 mμ) *vs.* time.

which the influence of the induction period was becoming less effective and the flocculation reaction was becoming more dominant. Each optical density–time curve showed a point of inflection which was interpreted to indicate that the flocculation process was essentially free of any induction period phenomena from this point to the time of complete reaction.

In a given experiment the optical density at time "t" is directly proportional to the quantity of protein flocculated (see Fig. 1 where the limiting optical densities are directly proportional to the concentration of protein, *i.e.*, the Beer–Lambert law applies). The slope of the linear section of the optical density–time curve after the induction period should then be directly proportional to the initial rate of flocculation. Numerical values of d(O.D.)/dt for the linear sections of the curves are given in Table II for different values of *p*H, chloroauric acid concentration and temperature.

TABLE II

DEPENDENCE OF RATE OF FLOCCULATION OF OVALBUMIN–CHLOROAURATE COMPLEX ON *p*H, TEMPERATURE AND $AuCl_4^-$ CONCENTRATION[a]

*p*H	Molar concn. of $AuCl_4^-$	Slope of linear section of optical density *vs.* time plot (d(O.D.)/dt) Temperature[b] 6.5°	20.5°	31.0°
3.47	0.00125	0.00269	0.00715	0.0240
	.00163	.00494	.0187	.0500
	.00250	.0164	.0583	.131
	.00375	.0802	.112	.360
3.88	0.00125	0.00601	0.0155	0.0502
	.00163	.0110	.0360	.0986
	.00250	.0445	.0763	.150
	.00375	.130	.233	.864
4.33	0.00125	0.014	0.0462	0.119
	.00163	.024	.0811	.159
	.00250	.065	.141	.403
	.00375	.381	.622	Too fast for measure.

[a] Ovalbumin concentration constant at 39.5 mg. per 100 ml. solution. [b] Temperature control, ±0.5°.

The data obtained from the optical density–time studies on the ovalbumin–chloroauric acid reaction may be summarized as follows, all other factors being constant

(a) the induction period
  (1) increased with decreased chloroauric acid concn.
  (2) increased with greater protein concn.
  (3) increased with lower temp.
  (4) increased with greater hydrogen ion concn.

(b) the limiting optical density
  (1) increased with lower hydrogen ion concn.
  (2) increased with greater chloroauric acid concn.
  (3) appeared to be temp. independent
  (4) was a linear function of the protein concn.

(c) the rate of flocculation
  (1) was roughly inversely proportional to the hydrogen ion concn.
  (2) was directly proportional to the logarithm of the protein concn. (slopes of linear sections of curves in Fig. 1 not tabulated)
  (3) was approximately proportional to the third power of the chloroauric acid concn.
  (4) was directly proportional to the quantity $e^{-1/T}$ where T is the absolute temp. Energy of activation values were grouped about 16,000 cal./mole

Light Scattering Experiments.—The light scattering studies described previously were designed to give additional insight into the above phenomena. Typical experimental results are shown in Figs. 2 through 5. The data presented in these curves were all taken under the same conditions of concentrations of reactants, *p*H, temperature and ionic strength. The induction period under the experimental conditions was approximately 170 minutes.

In Fig. 2 it is apparent that there is an initial rapid decrease in transmitted light which was only suggested in Fig. 1, in that in the latter, the optical densities did not have zero as their origin. Increased sensitivity and an expanded scale coupled

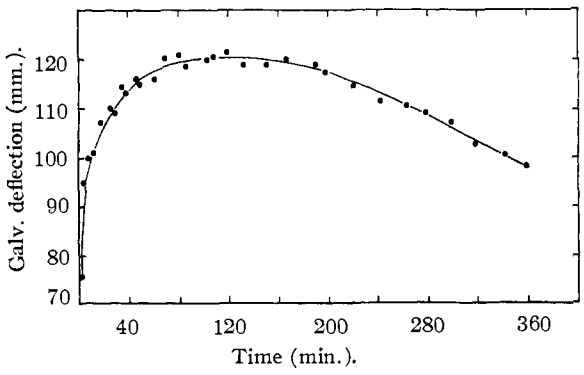


Fig. 3.–Intensity of transmitted light $U_{\mu 0}$ (λ 405 mμ) *vs.* time.

with optimum concentrations of reactants all contributed to making this fact more obvious from the data of Fig. 2.

Ovalbumin solutions do not appreciably absorb light of 405 or 547 mμ wave length. Chloroauric acid solutions do not absorb at 547 mμ but do absorb strongly at 405 mμ. Figure 3 shows an initial rapid increase in U_u^{405} with time and demonstrates that the first step in this rather complex process is the reaction of the protein with gold-bearing ions. Since the $AuCl_4^-$ ion is removed from solution in the over-all reaction it is presumed that this initial reaction is one of adsorption of these ions to the surface of the protein. The downward trend of the curves on Figs. 2 and 3 starting at about 170 minutes is associated with the initial formation of the final flocculant. A slight cloudiness of the suspension was observed visually 180 minutes after mixing.

The relationship between ρ_u, the ratio of H_u to V_u, and time is presented in Fig. 4. Neither H_u, V_u or ρ_u showed a plateau when plotted against time. These curves, however, were characterized by positive slopes and each showed a point of inflection during the induction period.

The beginning of the linear section of the ρ_u *vs.* time curve, point B in Fig. 4, coincides with the end of the induction period. The time interval between points A and B on this curve corresponds to the time interval of the plateaus in Fig. 2 and 3. One cannot really be certain whether the initial increase of ρ_u up to point A is to be attributed to increased intrinsic anisotropy of the protein–chloroaurate as compared with ovalbumin or to the greater anisotropy of the aggregates of these particles, for it seems that the processes are occurring almost simultaneously. Since, however, anisotropy is generally considered to be more a property of the molecule than of its size, we suggest that this initial increase represents primarily the increased anisotropy of the protein–chloroaurate. Between A and B it would seem then that the factor producing the increased anisotropy is changing from one intrinsic in nature to one determined almost totally by size and state of aggregation of the particles. Beyond point B the increased depolarization must be due primarily to flocculation of the protein–chloroaurate aggregates.

Scattering also increased as a function of time in the $I_{45}°$ and the $I_{135}°$ *vs.* time curves (not shown). The dissymmetry, Z, which is the ratio of these two values (Fig. 5) showed a rapid initial increase up to 1.6 in a period of about 14 minutes after mixing of the reactants. This was followed by a slower decrease in Z to a value of 1.35, where it remained sensibly constant until a time approximately corresponding to the end of the induction period. The dissymmetry steadily increased beyond this point.

The curve of Fig. 5 shows one of the only two dissymmetry runs which were made. Both experiments yielded data which plotted the same shaped curve as shown here. The fluctuation in Z occurring between 40 and 80 minutes on the time axis (shown by the dashed curve) appears to be real but no explanation seems to be readily available for this phenomenon. The general behavior demonstrated here, although unexpected, is not incompatible with a probable physico-chemical process which may be occurring in the system.

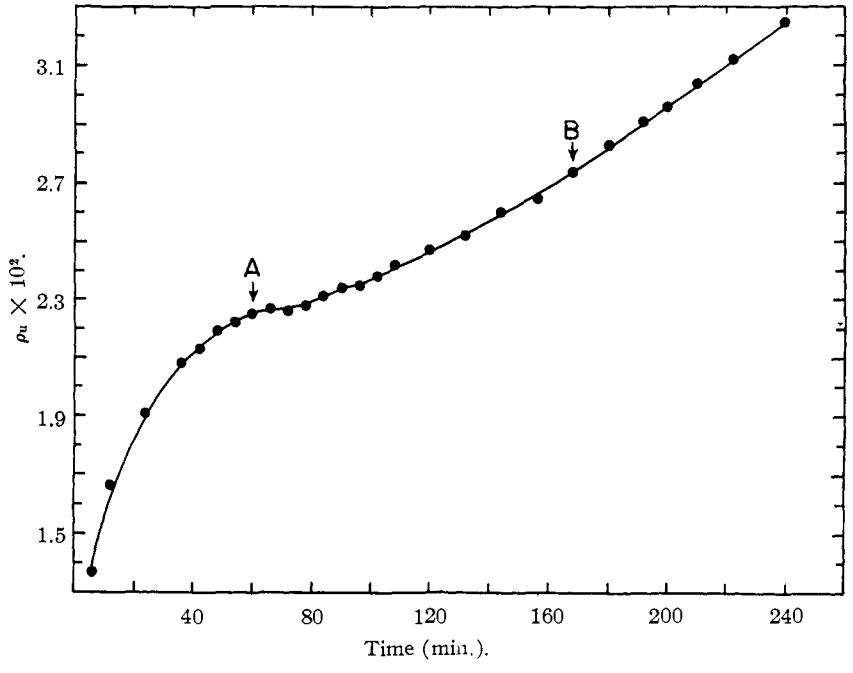


Fig. 4.—Depolarization ratio $\rho \times 10^2$ (λ 547 mμ) *vs.* time.

We consider the ovalbumin molecule to be an ellipsoid of revolution[14] roughly 30 by 90 Å. We can probably assume that effective collisions between molecules are more likely when long axes are parallel, and that as rapidly as the zeta potential is sufficiently lowered by reaction with $AuCl_4^-$

(14) H. Neurath, *Cold Spring Harbor Symposia on Quantitative Biology*, **VI**, 196 (1938).

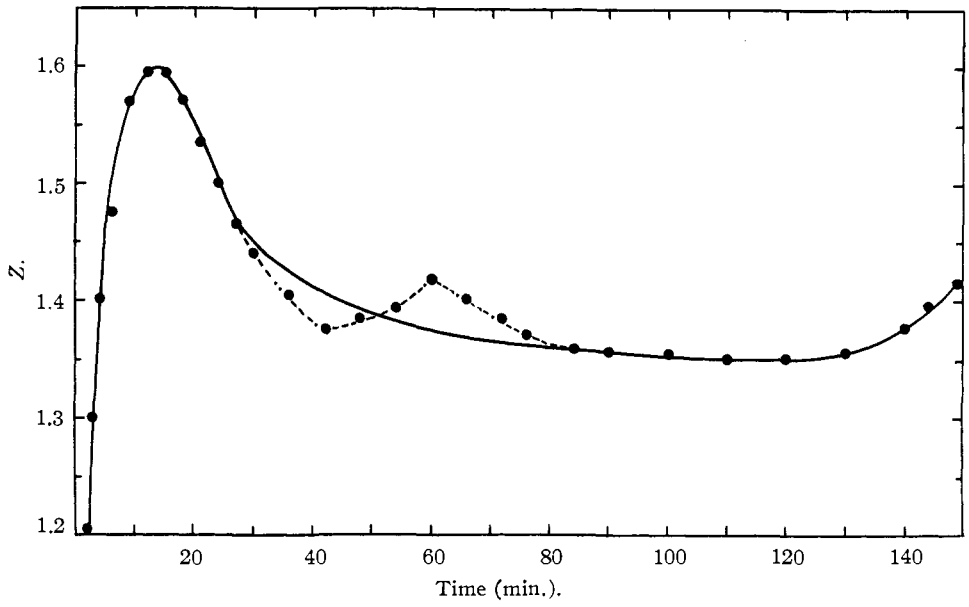


Fig. 5.—Dissymmetry ratio, Z, (λ 547 mμ) *vs.* time.

ion aggregation of the ovalbumin–chloroaurate complexes begins to occur.

We can, however, say little about the state of aggregation except that the maximum in the dissymmetry curve indicates that the initial aggregates have a preferred direction of growth or that they undergo a rearrangement such that the resulting secondary aggregate is characterized by a lower dissymmetry. One possible mechanism whereby angular scattering intensities would continue to increase with time and yet be compatible with the dissymmetry behavior would involve primary aggregates which are spherical but which rearrange into rods prior to flocculation. Such a behavior could result if spherical aggregation began before equilibrium was attained between the reacting groups of the protein and the $AuCl_4^-$ ions, whereas the rod-like configuration was more consistent with the ultimate stoichiometry of the system.

The process suggested above may be only one of several which would explain the experimental observations. It does show, however, that a consistent explanation of the data can be provided without an assumption of unfolding and denaturation. In fact, the latter interpretation would probably be incorrect in this case since the ovalbumin–chloroauric acid reaction liberates hydrogen ion and therefore proceeds more rapidly at the higher *p*H values (see Fig. 6 and Tables I and II). The denaturation of a protein on the acid side of its isoelectric point has been shown to be a process

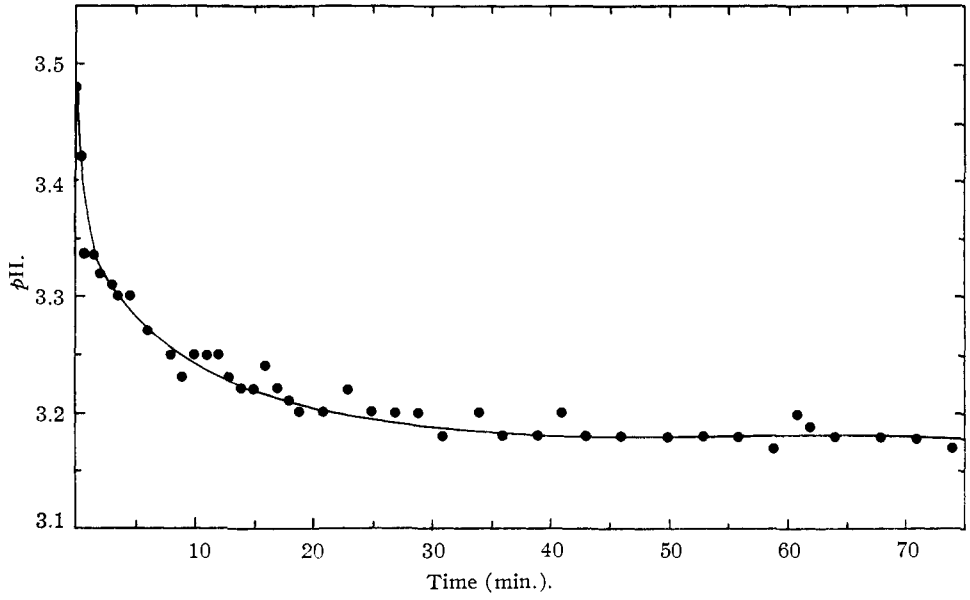


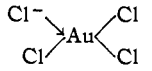
Fig. 6.—*p*H *vs.* time in unbuffered solution: protein concn., 0.33 mg./ml.; Au concn., 3.3×10^{-4} *M*.

which consumes hydrogen ion.[15] We believe the final flocculated ovalbumin–chloroaurate to be merely insoluble and chemically altered but undenatured protein.

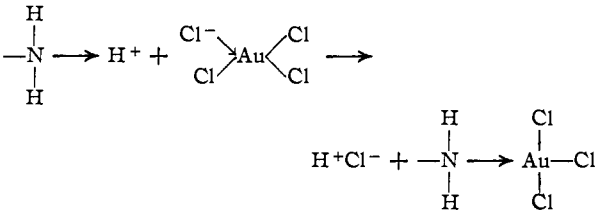
It was mentioned earlier that the *p*H was observed to decrease in unbuffered solutions. Figure 6 shows that hydrogen ion is liberated concurrent with the combination of the protein with the chloroauric acid. From this change in *p*H one can calculate the number of $AuCl_4^-$ ions which combine per molecule of ovalbumin, on the basis of the reasonable assumption that one hydrogen ion is released per $AuCl_4^-$ ion reacted. This calculation produced values ranging from 40 to 45 $AuCl_4^-$ ions per molecule of ovalbumin, corresponding to selected molecular weights of the protein ranging from 40,000 to 45,000. It is of interest to compare these values with the number of basic groups contributed by arginine, histidine, and lysine, which are given variously from 40 to 41 per ovalbumin molecule.[16]

The observation that the induction period is of longer duration at low *p*H values is now explained since hydrogen ion is produced in the reaction.

We turn now to the probable chemical reaction between amino groups and the $AuCl_4^-$ ion. The chloroaurate ion is itself a complex formed by $AuCl_3$ and Cl^- ion, *i.e.*



This ion is presumably first attracted to the positive $-NH_3^+$ centers on the surface of the protein by coulombic forces, and when it is sufficiently near, there is a splitting out of H^+Cl^- making the two electrons of nitrogen which were formerly coordinated with hydrogen ion now available for coördination with the gold of the $AuCl_3$. This produces $-NH_2 \cdot AuCl_3$ according to the scheme



This reaction is being investigated further in our laboratories in view of the possibility that chloroauric acid may serve as a reagent for the determination of the number of basic amino groups in a given protein molecule.

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(15) D. Lloyd and A. Shore, "The Chemistry of the Proteins," P. Blakiston' Sons and Co., Inc., Philadelphia, Pa., 1938, pp. 429–431.

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[CONTRIBUTION FROM THE SCHOOL OF CHEMISTRY, INSTITUTE OF TECHNOLOGY, UNIVERSITY OF MINNESOTA]

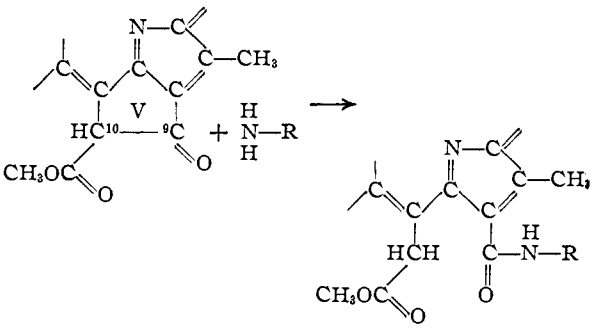
The Reaction of Chlorophyll in Amines[1]

BY ALBERT WELLER[2] AND ROBERT LIVINGSTON

RECEIVED OCTOBER 29, 1953

Chlorophyll dissolved in amines undergoes an irreversible reaction which involves cleavage of ring V. Reaction rates in different amines and at different temperatures were measured spectrophotometrically. The specific rates parallel the basicities of the amines. The energy of activation is very low, ~3 kcal. A mechanism analogous to the aminolysis of esters is suggested and discussed. It is suggested that the reported[3] phosphorescence of chlorophyll dissolved in isoamylamine, is very probably due to the chlorin-6-acidamide formed in the amine reaction.

In the course of investigations which have been carried out in this Laboratory on the photochemical behavior of chlorophyll and related compounds, it has been observed[4] that the absorption spectrum of chlorophyll is shifted to much shorter wave lengths (about 200 Å.) in amines. It cannot be ascribed to medium effects, but is due to a reaction product of chlorophyll[5] which is formed irreversibly in a reaction between chlorophyll and amine. Fischer and Göbel[6] using methylpheophorbide and different amines showed that it is the chlorin-6-acidamide which is formed by cleavage of ring V by the amine.



This reaction is of special interest, since Kautsky and co-workers[3] observed phosphorescence of solvents of chlorophyll ($-a + -b$) in isoamylamine but not when other (non-amine) solvents were used. It seems probable, therefore, that the phosphorescence which those authors observed was due to chlorin-6-acidamide rather than to chlorophyll.

Krasnovskii and Brin[7] attribute the difference,

(1) This work was made possible by the support of the Office of Naval Research (NR 051,028, Contract N6ori-212, T. O. I.) to whom the authors are indebted.

(2) Post-doctoral Fellow 1951–1952. Present address: Laboratorium für physikalische Chemie, Widerholdstr. 15, Stuttgart, Germany.

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