to an acidic compound. This treatment did not alter the biological activity of the isolated peptide significantly (195 units per mg. before hydrolysis and 170 units per mg. after) or change the electrophoretic behavior on paper between *p*H 1 and 8. The fact that the original peptide contained glutamic acid and not glutamine was also implied by the fact that it was electrophoretically neutral at *p*H 5.0. The corresponding glutamine peptide should have been basic. A more positive way of showing that the peptide contained glutamic acid in preference to glutamine would have been by hydrolysis with carboxypeptidase, followed by chromatographic identification of glutamic acid or glutamine. However, carboxypeptidase did not attack this peptide.

Discussion

Since this peptide survived the action of pepsin, trypsin, chymotrypsin, carboxypeptidase and dilute acid, it should be a suitable material with which to investigate the strepogenin requirement of growing animals.[22,23] Sufficient quantities for such an experiment could be obtained only through chemical synthesis.

As was the case with the acid digest of insulin,[3] many peptides with strepogenin activity were present in enzymic hydrolyzates (see Fig. 1). That more than one arginine-containing peptide with biological activity was present was shown in Figs. 2 and 3. Because these materials appeared to be present in even smaller amounts than the peptide isolated, it seemed impractical to attempt to isolate them. The peptide isolated in pure form accounted for 4.2% of the strepogenin activity of the enzymic hydrolyzate of insulin. Undoubtedly, only a fraction of the peptide found in the hydrolyzate was isolated in pure condition.

Sanger and Tuppy,[24] using a peptic digest of the oxidized B-chain of insulin, identified the peptide leucylvalylcysteylglycylglutamylarginylglycylphenylalanine[25] (Bp4) as a spot on paper chromatograms. Later, from unoxidized insulin, Sanger, *et al.*,[26] obtained the unsymmetrical disulfide involving leucylvalylcysteinylglycylglutamylarginylglycylphenylalanylphenylalanine and cysteinylaspartic acid. The structure of this disulfide was deduced from a study of the products of the oxidation with performic acid. This sequence was confirmed by Haugaard and Haugaard[27] who obtained a fraction, the amino acid composition of which corresponded to the unsymmetrical disulfide involving leucylvalylcysteinylglycylglutamic acid and cysteinylaspartic acid by chromatographing subtilisin-digested insulin on Dowex-50 x 4. In neither case was the proposed structure based on an isolated peptide of proved purity. The present work, which deals with analytically-pure material, confirms the amino acid sequence from leucine to arginine.

All the evidence indicated that material isolated in the present work was the symmetrical disulfide. This disulfide is not a part of the structure of insulin as proposed by Sanger and co-workers.[26] However, one can picture the formation of this symmetrical disulfide from disulfide interchange[13] of the unsymmetrical peptides deduced by Sanger. If the Sanger structure for insulin is correct, one must assume that this disulfide interchange occurred and was responsible for the formation of the peptide herein described.

Although it is commonly held that protein digests prepared by the combined action of several enzymes are too complex to permit isolation of pure peptides by the use of existing methods, the present work shows that this is not necessarily so, when several columns of different ion-exchange resins are used sequentially.

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Solubility and Mechanism of Dye-uptake in Protein–Dye Salts[1,2]

BY DONALD B. WETLAUFER[3] AND MARK A. STAHMANN

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The precipitation of the basic protein lysozyme by the acidic dye, Orange II, has been studied systematically. From a titration of dye into the protein solution and analyses for dye in filtrate samples at frequent intervals, a plot is constructed of dye uptake as a function of free dye concentration. This graph summarizes quantitatively the solubility and stoichiometry of the precipitate. A reversible equilibrium exists between the solution and the amorphous precipitate. The precipitate continues to bind dye after lysozyme is completely precipitated from solution, up to an extrapolated maximum value which at *p*H's less than 4 is equal to the number of positively charged groups in lysozyme. The mutual precipitation of lysozyme and Orange II shows quantitative agreement with a solubility-product model, while the further binding of dye by the precipitate can be described as a simple association where all the dye-binding sites have the same binding energy. The lysozyme–Orange II system was studied with temperature, *p*H, ionic strength and initial protein concentration as experimental variables.

The ability of ionic dyes to precipitate proteins from solution has long been known. Although such diverse substances as colloidal metal oxides, tannic acid, polyphosphates, ionic detergents and trichloroacetic acid are also effective protein precipi-

(1) Part of a thesis presented to the Graduate School of the University of Wisconsin by D. B. Wetlaufer in partial fulfillment of the requirements for the Ph.D. degree, 1954.
(2) Published with the approval of the Director of the Wisconsin Agricultural Experiment Station. A preliminary report of this work was presented at the 125th meeting of the American Chemical Society, Kansas City, Mo., March 25, 1954.
(3) Predoctoral Fellow of the National Science Foundation, 1952–1954.

tants, ionic dyes possess two special advantages for experimental studies: they can be obtained as chemical individuals of known composition and purity and by virtue of their intense colors they can be analyzed readily by photoelectric colorimetry.

The salt-nature of the combination between proteins and ionic dyes was established by Schmidt and co-workers[4,5] on the basis of the correspondence between the amount of dye bound and the number of H^+-binding groups in proteins. These results provided the basis for a method by Cooper and Fraenkel-Conrat[6] for determining the available acidic and basic groups in protein samples by determining the maximum amount of uptake of dye from an added solution.

The present work was undertaken with the long-range goal of determining whether the precipitation of proteins as dye-salts could provide the basis for an analysis for the components of protein mixtures. We have here determined the amounts of protein and dye both in solution and in the precipitate, over a wide range of initial reagent concentrations. This is conveniently done by titrating a dye solution into the protein solution and measuring the soluble dye at intervals. The response of the system to controllable variables, such as *p*H, salt concentration and temperature, was also of interest.

In a previous study,[7] we found that the solubility behavior of such a system can be summarized on a co-ordinate system showing dye precipitated as a function of the dye concentration in solution. This way of representing the concentration of the added precipitant throughout the course of a precipitation falls within Toffoli's generalization[8] or riptographic analysis, and accordingly, we will refer to such plots here as riptograms.

The following experiments were carried out with the basic protein lysozyme, which is a desirable choice for several reasons: it is a remarkably stable,[9] readily available protein, easily obtained at a high degree of purification. Its amino acid composition is fairly well established,[10,11] and its acid-base properties have been thoroughly studied.[12] The precipitation reagent, Orange II, is a stable, inexpensive, readily purified dye, which precipitates lysozyme from solution at a low dye concentration.

Experimental

Materials.—Lysozyme was purchased from Armour and Co., Chicago, Illinois (lot number 003L1). The supplier's analyses indicate 4.12% moisture, ash (sulfated) 1.52%, nitrogen 18.3%. A single electrophoretic analysis of this lot of lysozyme (by the Armour Laboratories) at *p*H 7.7 (phosphate buffer) and ionic strength 0.20 showed two components with mobilities 1.82 (95%) and 0.60 (1–5%) $\times 10^{-5}$ cm. sec.$^{-1}$ volt^{-1}. Tallan and Stein suggest[13] that the usual presence of about 5% electrophoretic impurity in lysozyme is due to the hydrolysis of some of the amide groups of the protein. The mobilities reported in the above analyses are lower than those found by Tallan and Stein at nearly the same *p*H in Veronal buffer. However, it has been noted[10] that lysozyme exhibits an abnormally low mobility in phosphate buffers. A mean molecular weight of 14,700 from several determinations[10–15] was employed for the lysozyme chloride salt.

Stock solutions of lysozyme were prepared to a concentration of 0.5% in glass-distilled water. These solutions were stored in the frozen state at $-15°$ and aliquots taken as needed by thawing and withdrawing samples through a sintered-glass filter stick (the solution was clarified readily by this filtration). That this method of storage has no measurable effect on the solubility of lysozyme is attested by the agreement of duplicate analyses of the solute content of the same stock solution after a three-month interval which included about ten thawing-freezing cycles. The concentration of these stock solutions is determined by drying an aliquot *in vacuo* at 105–110°. The concentration of lysozyme is corrected for ash content on the assumption that the impurity is entirely NaCl (lysozyme is crystallized from NaCl solutions) in an amount equivalent to 1.52% Na_2SO_4. We determined ϵ at 281 mμ to be 3.90×10^4 in *p*H 3.9 formate buffer, in good agreement with the value of 3.88×10^4 in 1/10 *N* HCl reported by Fromageot and Schnek.[16]

Orange II, which is the monosodium salt of *p*-(2-hydroxy-1-naphthylazo)-benzenesulfonic acid, was obtained from Eastman Kodak Co. as a recrystallized product. It was purified by extraction with a small volume of water, followed by recrystallization from water. Orange II has an absorption maximum at 485 mμ and a minimum near 281 mμ. We have determined ϵ 2.33×10^4 from absorbancy measurements in $\Gamma/2$ 0.10 formate buffer at *p*H 3.0. This value was obtained by extrapolating to infinite dilution a plot of ϵ *vs.* absorbancy. This curve was linear with a slight negative slope. At 281 mμ, we found ϵ $6.0_0 \times 10^3$. Separate tests showed no change in the absorbancy of Orange II at 450, 485 and 515 mμ in the *p*H range from 3.1 to 6.2. Stock solutions of Orange II in glass-distilled water were prepared directly from the recrystallized dye as furnished by the manufacturer.

Buffers were composed as follows: at *p*H 2.3, glycine + HCl; *p*H 3.2, sodium formate + HCl; *p*H 3.9, sodium formate + HCl; *p*H 4.6, sodium acetate + acetic acid; *p*H 6.4, primary + secondary sodium phosphates. The buffer components were of reagent grade.

Methods.—Enzymatic assays for lysozyme were carried out by a modification of the method of Smolelis and Hartsell.[17] The modification consisted of making the turbidity measurements at 580 mμ (which reduces the absorbancy by Orange II to a negligible level) instead of 540 mμ as recommended by these authors. We used as substrate dried, killed cells of *M. lysodeikticus*, supplied by the Difco company, Detroit, Michigan. The presence of Orange II at concentrations encountered in the assays was shown not to interfere with the assay method.

Absorbancies in the visible range were measured with a Beckman Model B Spectrophotometer, measurements in the ultraviolet were made with a Beckman Model DU Spectrophotometer. Early measurements of dye concentrations were made in standard 10 mm. cuvettes, but their use was superseded by thinner cells designed to facilitate the filling and emptying operations and to minimize losses in a series of observations on a single sample. The cell is essentially a bulb-operated pipet mounted in a fixed position on the photometer cell carrier.

Reaction Vessel.—The precipitation reaction is conveniently carried out in a two-necked 125-ml. Pyrex flask, one neck for a micro-buret and one for a filter-stick. Stirring without foaming is achieved with a Teflon-coated

(4) L. M. Chapman, D. M. Greenberg and C. L. A. Schmidt, *J. Biol. Chem.*, **72**, 707 (1927).

(5) L. M. C. Rawlins and C. L. A. Schmidt, *ibid.*, **88**, 271 (1930).

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(17) A. N. Smolelis and S. E. Hartsell, *J. Bact.*, **58**, 731 (1949).

stirring bar. To carry out a precipitation isothermally, the flask is immersed in a bath thermostatically controlled to within ±0.10°.

Filtration.—The use of a filter stick allows isothermal filtrations to be carried out. The filter sticks are 10 mm. Corning Pyrex fine-porosity sintered glass. They are made with a thick-walled capillary stem, which adds to their durability and decreases the volume of the filtrate required. At the top of the filter stick is a ground-glass joint which serves also as a reservoir from which filtrate can be withdrawn for photometric analysis. There is a small uptake of Orange II from solution by the sintered-glass filter, of the order of 1% decrease in the optical density of 40 ml. of solution. A correction curve can be made for a given temperature, solvent and filter stick, plotting moles of dye taken up *vs.* the equilibrium concentration of dye. This correction is more precisely determined in a small volume of solution. For work of the highest precision, *e.g.*, stoichiometry, this correction must be applied. We find that lysozyme was taken up from *p*H 3.9 formate–HCl buffered solutions to an extent of less than 1% when the lysozyme concentration was ten times greater than that usually employed in these experiments. This error was assumed to be negligible.

The equilibration of an Orange II solution with the sintered glass filter is effectively complete in about ten minutes when the dye solution is pumped back and forth across the filter element two to three times per minute.

Delivery of the Dye Reagent.—The concentrated Orange II solution is delivered into the stirred lysozyme solution with a microburet, with which volumes can be measured by interpolation to 10^{-4} ml. The buret is air-thermostated at a temperature constant within ±1°. Orange II solutions are preferably made up in water, not in buffer, because of the electrolyte limitations on the solubility of this dye. Normally the addition of the total volume of dye solution increases the volume of the reaction solution by about 1%, so the ionic strength and protein concentration are not changed appreciably by dilution. However, this volume change is accounted for in determining the amount of soluble dye.

Protocol of a Typical Riptographic Analysis.—An aliquot of a lysozyme stock solution is transferred to a reaction vessel containing a volume of solvent such that the final volume is 40.0 ml. Three aliquots of Orange II solution are delivered from the microburet into known volumes of the same solvent, for colorimetric standardization. The absorbancy of the solvent is determined (*vs.* an air blank). The stirring is begun in the reaction flask and the first aliquot of dye is added to the solution. The equilibration pump is connected to the filter stick. After 12 minutes the filter stick is coupled with a rubber bulb and filtrate drawn up into the reservoir. The observation cell is filled with filtrate from the filter stick reservoir and fitted into the colorimeter and the absorbancy measured (*vs.* an air blank). The cell assembly is removed from the colorimeter and the filtrate returned from the cell to the reservoir, forced (by a rubber bulb) from the reservoir back into the reaction flask, and another aliquot of Orange II is added to the reaction mixture. The air pump is reconnected to the filter stick. The observation cell is cleaned with alkaline detergent, thoroughly rinsed with water and then with acetone and is dried with a stream of filtered air after detaching its rubber bulb. Dye additions can be made conveniently at 15-minute intervals. After the completion of the analysis (10–20 aliquots of dye), three more aliquots of Orange II are delivered for standardization, and the concentrations of all the standards are determined.

The details of the calculations are available elsewhere.[18] For plotting the results, the coördinate system "Δ Optical density" *vs.* "Optical density" is linearly related to the system "Moles Orange II precipitated" *vs.* "Orange II molarity," and the two systems can be used interchangeably.

Results

Equilibration.—The effect of increasing the time of equilibration is shown in the riptograms of Fig. 1. On aging, the precipitate becomes less soluble, but the general form of the riptogram remains unchanged. In this coördinate system, individual ob-

(18) G. Scatchard, THIS JOURNAL, **72**, 538 (1950).

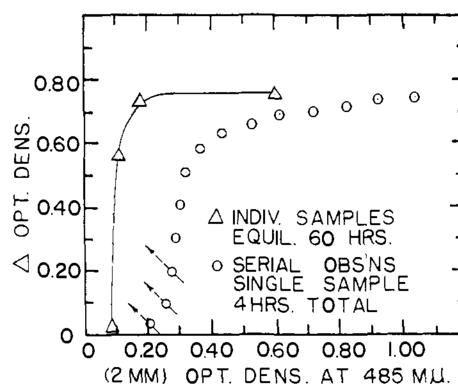

Fig. 1.—Effect of time on the solubility of a lysozyme–Orange II precipitate: Δ, individual samples, equilibrated 60 hr.; O, serial observations on a single sample, 15 min. intervals: 15°, *p*H 3.95, Γ/2 0.10 formate–HCl buffer, initial lysozyme concentration 9.9 × 10^{-6} molar.

servations will shift with time along a route of slope −1, as suggested by the arrows.

To estimate the rate of approach of such a system to final equilibrium, we observed several reaction mixtures to which sufficient Orange II had been added (in one aliquot at zero time) to result in about 50% maximal dye precipitation, when measured within 1 hr. Figure 2 shows the results of

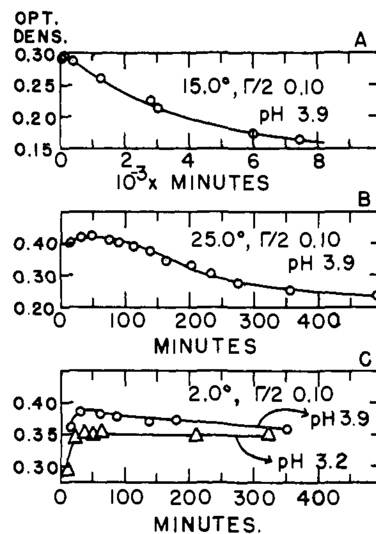

Fig. 2.—Solubility as a function of temperature: Orange II absorbancy *versus* time. In every case, sufficient Orange II was added to precipitate about half the lysozyme, whose initial concentration was 9.9 × 10^{-6} molar. Absorbancies were measured through 2 mm. sample path.

these experiments at different temperatures. In Fig. 2a it is seen that equilibration is incomplete after five days at 15°. At 25° (Fig. 2b) this process is much accelerated, but measurements at 1440 and 1680 minutes (not plotted here) show that equilibrium has not yet been reached. The experiments of Fig. 2c show that an approximately steady period is found between 30 and 300 minutes at 2°. From these experiments we are led to believe that the time of achieving a final equilibrium in the lysozyme Orange II system is prohibitively great at temperatures between room temperature

and the ice point. While the rate of equilibration is greatly accelerated by temperature, there are several factors militating against higher temperatures. First, the positive temperature coefficient of solubility of the precipitate means that filtrates from reactions above room temperature will be unstable with respect to further precipitation when removed for colorimetric analysis. In addition the likelihood of bacterial or mold growth and protein denaturation increases with rising temperature. These considerations led us to examine the possibility of using the steady state referred to in Fig. 2c instead of the final equilibrium state.

Reproducibility.—In Fig. 3 are shown riptograms obtained from two experiments where serial

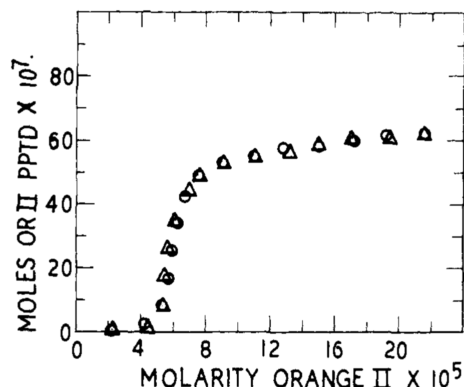

Fig. 3.—Reproducibility of lysozyme–Orange II riptograms. Parallel experiments at pH 3.97, $\Gamma/2$ 0.10 formate–HCl buffer, 15.0°. Time interval between successive additions of Orange II: O, 15 min.; △, 30 min. Initial lysozyme concn. 9.9×10^{-6} molar, reaction volume 40.0 ml.

additions of Orange II were made to single lysozyme samples, in one case at 15-minute intervals and in the second case at 30-minute intervals. Although the curves of Fig. 2 show a small time-dependence for the dye-uptake in the steady state, the effect appears much larger in Fig. 2 than in the actual riptograms. The results of Fig. 3 show that there is very small difference between 15-minute and 30 minute-intervals at 15°. The results of Fig. 2 lead us to expect even smaller differences at 2°; this

TABLE I

TEST FOR REVERSIBILITY OF LYSOZYME–ORANGE II PRECIPITATE

Temperature 2.0°, pH 3.2, $\Gamma/2$ 0.10, final volume 40 ml. in both cases.

Twofold diln. at 83 min.		Diluted to final vol. at zero time	
Time from initial mixing, min.	Orange II pptd., moles $\times 10^7$	Time from initial mixing, min.	Orange II pptd., moles $\times 10^7$
24	16.15	25	11.33
49	16.05	54	11.47
65	16.08	96	11.49
98	11.45	146	11.41
124	11.45	218	11.39
155	11.37		
240	11.43		
Initial lysozyme concn. 9.9×10^{-6} m		Initial lysozyme concn. 4.95×10^{-6} m	

is also supported by the experiments shown in Table I. From the good agreement of these two sets of experiments, it appears that the steady state is reproducible over a wider time-range than was customarily employed in our experiments.

Reversibility.—To test the reversibility of the Orange II–lysozyme precipitate in the steady period, we conducted an experiment in which a series of observations of Orange II concentration were made in filtrates of a reaction mixture, and after some 80 minutes of stirring the whole system was diluted by adding isothermally an equal volume of solvent. The observations of Orange II concentration were continued. A control experiment was run in which the dye was added directly to the protein solution at the final dilution. The results of this experiment are shown in Table I. Dilution of the system results in re-solution of precipitate and the establishment of a new steady condition within 15 minutes. The amount of dye precipitated is the same whether by dilution or by direct addition. These results support the idea that there is a rapid equilibration between precipitate and solution in the steady state.

Stoichiometry.—By carrying out solubility experiments in which both Orange II and lysozyme are analyzed in the filtrates, stoichiometric relationships were determined. Six separate reaction mixtures were filtered after an hour's equilibration, Orange II was analyzed by its visible absorbancy, and lysozyme was analyzed both by its ultraviolet absorbancy and by its lytic activity. The results are shown in Fig. 4. There is a small uncertainty

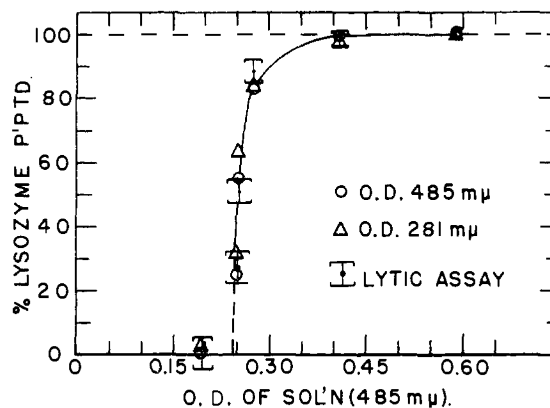

Fig. 4.—Comparison of lysozyme determination: O, by difference in Orange II absorbancy at 485 mμ; △, by lysozyme absorbancy at 281 mμ; bracketed points, by enzymatic assay of filtrate (distance spanned by brackets represents uncertainty of the measurement). In all cases the abscissa depends solely on the absorbancy measurement at 485 mμ. pH 3.95, $\Gamma/2$ 0.10 formate–HCl buffer, 15.0°.

in deciding from a plot of Δ absorbancy (485 mμ) $vs.$ absorbancy (485 mμ) just where the lysozyme precipitation is complete, since dye continues to be removed from solution even after all detectable lysozyme has been precipitated. We have adopted the empirical procedure of taking the ordinate of a riptogram at an abscissa value twice that at the estimated center of the steeply-rising portion of the riptogram as representative of complete lysozyme precipitation, when riptograms are based solely on

Orange II analyses.[19] This particular uncertainty does not arise in the direct analysis for lysozyme, and it will be seen that there is a strong parallel in the ordinates obtained by the different analytical methods. These results also show that lysozyme in equilibrium with the lysozyme–Orange II precipitate retains its enzymatic activity, which again indicates the reversible nature of this precipitation process.

To obtain further evidence in the matter of stoichiometry, another series of experiments was carried out, essentially the same as those just described except that the amounts of Orange II added were chosen so that the resulting points on a riptogram would all fall on the steeply-rising part of the curve. Both the dye and the protein were determined by absorbancy measurements on filtrates. The results, as plotted in Fig. 5, show a

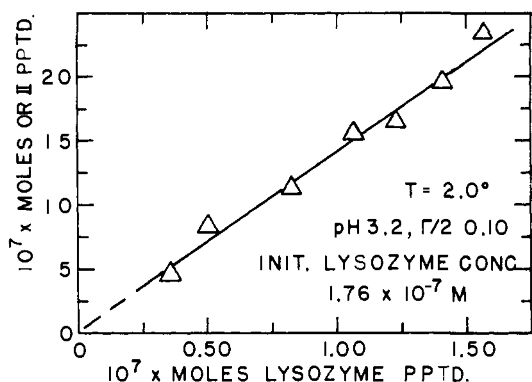

Fig. 5.—Test of the constancy of composition of lysozyme–Orange II precipitate, in the range of mutual precipitation. Each point represents a separate sample, analyzed after 60–90 min. equilibration: 2.0°, *p*H 3.15, Γ/2 0.10, formate-HCl buffer, initial lysozyme concentration 1.76×10^{-5} molar, reaction volume, 10.0 ml.

linear relationship between the moles of Orange II precipitated and the moles of lysozyme precipitated, demonstrating the constancy of composition of the precipitate throughout the process of mutual precipitation. The slope of this curve is 14.1 moles of dye per mole of protein, compared with a value of 14.5 obtained from a separate experiment under the same conditions when only Orange II was analyzed.

The stoichiometry is also reflected in comparing a family of riptograms where only the initial amount of lysozyme is varied. Such a comparison is afforded in the experiments of Fig. 6. Estimating the ordinate for complete lysozyme precipitation as previously described, we find values of moles of dye per mole of protein to be 13.6, 12.9, 13.1 and 13.4 for the curves labeled 1.5, 3, 6 and 12 mg., respectively.[20] Making the same estimate for the

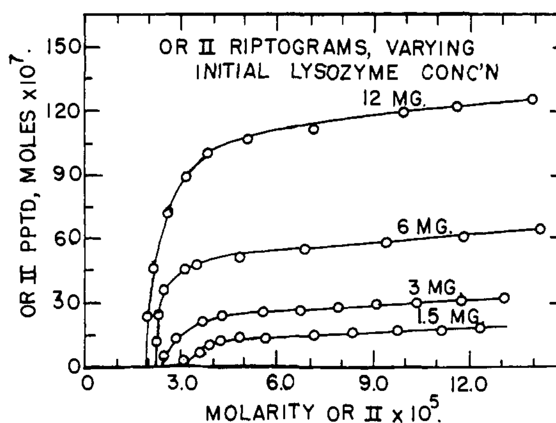

Fig. 6.—Lysozyme–Orange II riptograms, varying initial lysozyme concentration: 2.0°, *p*H 3.97, Γ/2 0.10, initial volume 40.0 ml. Exact amounts of lysozyme: 1.45 mg., 2.91 mg., 5.82 mg., 11.64 mg.

riptograms of Fig. 3, which were obtained in the same solvent but at 15°, we find a dye/protein molar ratio of 13.4. From a comparison of experiments at these two temperatures there is no indication of a temperature effect on the precipitate composition, although there is about a 70% increase in solubility at the higher temperature.

Effect of Ionic Strength.—The effect of varying the ionic strength of the solvent at constant *p*H is shown in Fig. 7. The salting-out effect of electrolytes on Orange II prevents study at ionic strengths much greater than 0.20. The solubility of the precipitate increases with increasing ionic strength, but no significant change in the stoichiometry is apparent.

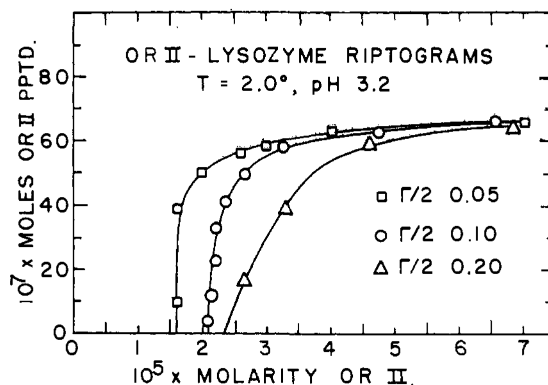

Fig. 7.—Effect of ionic strength on lysozyme–Orange II riptograms: □, Γ/2 0.05; O, Γ/2 0.10; Δ, Γ/2 0.20: 2.0°, *p*H 3.15, formate–HCl buffer. Initial lysozyme concentration 9.9×10^{-6} molar, volume 40.0 ml.

Effect of *p*H.—A series of riptograms showing the effect of varying *p*H with ionic strength constant in the lysozyme–Orange II precipitation is shown in Fig. 8. The stoichiometry determined from these riptograms is listed in Table II. As the acidity is decreased, the precipitate becomes more soluble, and the ratio of dye to protein decreases. Specific ion effects are not excluded from these results, but since the change in solubility and stoichiometry is monotonic with *p*H, such effects, if they exist, are probably of minor importance.

(19) This procedure has basis in the calculation according to the solubility product principle, that a precipitation reaction $A + 10B \rightleftarrows AB_{10}$ is 99.5% complete when the concentration of B is 1.6 times as great as its value at 50% completion. The use of a concentration 2 times the 50% precipitation level makes allowances for possible deviations of a riptogram from an ideal form but may tend to give slightly higher than true values because of dye-binding by the precipitate.

(20) It may be noted that the concentration of 12 mg. of lysozyme per 40 ml. of solvent is close to the upper limit of our present methods, since the rate of filtration is markedly reduced when this quantity of lysozyme is precipitated.

TABLE II
STOICHIOMETRY OF LYSOZYME–ORANGE II PRECIPITATES AS A FUNCTION OF pH

pH	Moles Or. II[a] / Moles lysoz.
2.33	16.0
3.15	14.6
3.93	13.1
4.60	11.4
6.40	9.0

[a] Determined from the riptograms of Fig. 8, by the method described in the text.

Discussion

Since interpretation of our experimental results relies so heavily on the assumption of efficient filtration, we shall consider this question first. Suppose that small aggregates of precipitate pass through the filter and dissolve at room temperature to yield an increased absorbancy in the observation cell. If this made a significant contribution to the measured absorbancies, we should expect to obtain more erratic results in duplicate experiments (compare Fig. 3). An alternative is that the filtration failure is real but reproducible at any given ratio of dye to protein. Then, since the amount of precipitate in the solution progressively increases during a titration, this will decrease the operating size of the filter pores,[21] so we should expect to have a progressive increase in filtering efficiency if there is a continuous distribution of aggregate sizes. This will have the effect of displacing the lower part of the steeply-rising segment of the riptogram further to the right than the upper part, the net result being an S-shaped curve. The anomaly of the S-shaped curve has been considered in detail elsewhere.[22] Such an effect has been observed experimentally in a number of instances.[1,2,6] Since this effect is not found in our results and since our experimental observations are readily reproducible with different filters, the filtration used here can be considered efficient.

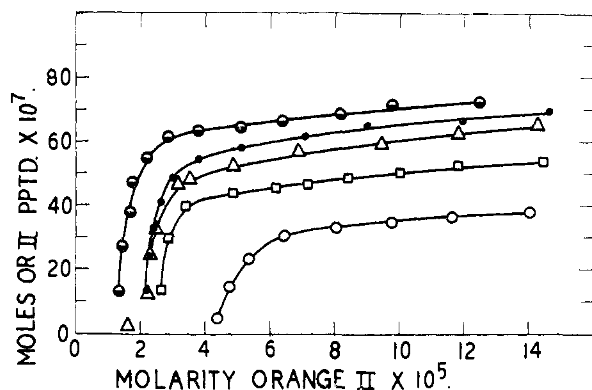

Fig. 8.—Riptograms for the precipitation of lysozyme by Orange II at differing pH's: O, pH 6.4; □, pH 4.60; △, pH 3.97; ●, pH 3.15; ◒, pH 2.33. Temperature 2.0°. Γ/2 0.10 for all experiments. Initial lysozyme concentration 9.65 × 10^{-6} molar, except at pH 3.15 and pH 3.95 concentration 9.9 × 10^{-6} molar; initial volume 40.0 ml.

(21) This is evidenced experimentally by a drop in filtration rate.
(22) D. B. Wetlaufer, Ph.D. Thesis, University of Wisconsin, 1954.

Dye Uptake by Lysozyme–Orange II Precipitate.—It is apparent on inspection of the experimental riptograms that a truly horizontal plateau of dye-uptake (*cf.* Figs. 3, 6, 8) is not attained after the steeply-rising part of the curve is passed. From the stoichiometric analyses (*cf.* Fig. 4) we know, however, that the lysozyme is completely precipitated at a dye concentration no more than twice that of the steeply-rising part of the riptogram. Since mutual precipitation cannot be responsible for this continued dye uptake, we propose that the mechanism is an ion-exchange process between buffer anions and Orange II anions at cationic sites of lysozyme. Assuming that all the dye-binding sites of lysozyme have the same apparent dissociation constants and that electrostatic effects are negligible at the ionic strengths employed, we can think of describing the binding of dye by the precipitate in simple mass-action terms. We have tested this by plotting the data from the plateaus of several riptograms in the form of the mass-action equation employed by Scatchard[18]

$$\bar{v}/c = (m - \bar{v})/k'$$

where $\bar{v}$ is the average number of molecules of Orange II bound per molecule of lysozyme, c is the molar concentration of Orange II, m is the maximum number of binding sites per molecule of lysozyme and k' is the apparent dissociation constant. The results of these plots of our data are seen in Fig. 9 to fit well to linear approximations. The values of m obtained by extrapolation of these plots are shown in Table III.

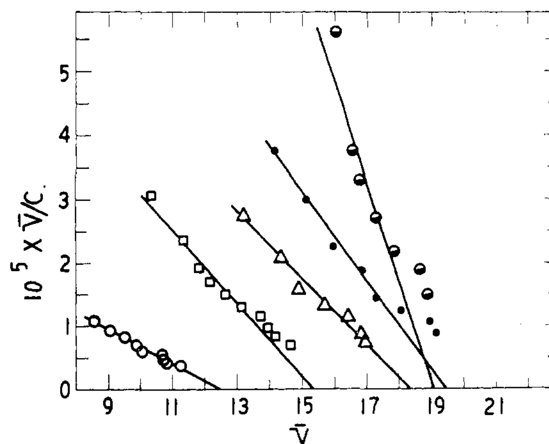

Fig. 9.—Plots of Orange II-binding by the lysozyme–Orange II precipitate. See text for explanation of coordinates. Point designation and experimental conditions, same as Fig. 8.

The extrapolated values for the pH 2.3 and the pH 3.2 experiments fall very close to 19, the number of positively-charged groups per lysozyme molecule according to amino acid analyses and acid–base titrations.[10-12] At the higher $\bar{v}$ values in all these plots there is some tendency for curvature which would extrapolate to higher values of m. This could be interpreted as indicating the existence of additional binding sites with a lower affinity for Orange II. The highest values of $\bar{v}$ in these plots result from observations at high absorbancies and

TABLE III

MAXIMUM ORANGE II BOUND IN THE PROTEIN–DYE PRECIPITATE, AS A FUNCTION OF *p*H

*p*H	Moles Or. II[a] / Moles lysoz. max.
2.33	19.1
3.15	19.4
3.93	18.2
4.60	15.3
6.40	12.6

[a] From the intercepts of Fig. 9.

were made near the end of a series of observations, where cumulative errors will be the greatest. For these reasons these are less reliable data, and we do not consider them to be sufficient evidence for additional binding sites. In drawing lines for extrapolation we have in all cases weighted these points less heavily.

The extrapolation of Orange II uptake to values less than 19 at higher *p*H's certainly is real, and we are led to speculate that within the precipitate some negatively-charged carboxylate groups of lysozyme are much better competitors for combination with the positive lysozyme sites than is Orange II. In the acid titration of lysozyme,[13] 10 equivalents of H^+ are taken up between *p*H 6.4 and *p*H 2.3. This value is considerably higher than the difference of 7 equivalents of Orange II found between these two *p*H values for both the stoichiometry (Table II) and the extrapolated value of m (Table III). Thus there does not seem to be any immediate connection between the binding of Orange II and that of H^+ through this *p*H-interval.

In the limited number of cases where comparison is possible, changing the ionic strength or the temperature has no significant effect on the value found for m.

Cooper and Fraenkel-Conrat, using their dye precipitation method,[6] found 88% of the basic groups in lysozyme,[12] 70% of the basic groups in gelatin[23] and only 55% of the basic groups in insulin.[24] We believe that these low results may be in part due to the failure to saturate these proteins with dye even at high dye concentrations; that is, a horizontal plateau may never be reached experimentally. The extrapolation method here employed for determining the basic groups in lysozyme may be generally useful in dye-precipitation methods for determining ionizable groups in proteins. This contention is supported by the recent studies on the binding of Orange G to collagen.[25]

Salt-nature of the Precipitate.—The salt-like nature of the lysozyme–Orange II precipitate is strongly supported by the correspondence[1,2] between the maximum number of Orange II anions bound and the number of positive charges on the lysozyme molecule. We have chosen a solubility-product model for comparison with the experimental riptograms. To be rigorously applied the solubility-product argument requires a homogeneous solid phase. We have shown (Fig. 5) that there is no detectable change in the composition of lysozyme–Orange II precipitate throughout the course of its progressive precipitation. However, due to the superimposition of the binding of dye by the precipitate on the binding due to mutual precipitation, there must be a small but real increase in the relative Orange II content of the precipitate as more and more lysozyme precipitates. Even so, the solubility-product model still appears to be the most suitable of simple models.

For a precipitation reaction $\alpha A + \beta B \rightarrow A_\alpha B_\beta$, where α and β are the stoichiometric coefficients corresponding to the reactants A and B, the concentrations of reagents in equilibrium with $A_\alpha B_\beta$ will be found by the solubility product relationship

$$K_{sp} = \alpha_A{}^\alpha \alpha_B{}^\beta = [A]^\alpha [B]^\beta \gamma_A{}^\alpha \gamma_B{}^\beta$$

where the bracket quantities refer to molar concentrations, α is activity and γ is the activity coefficient. If we assume that because of a swamping concentration of buffer ions the product $\gamma_A{}^\alpha \gamma_B{}^\beta$ remains constant throughout the mutual precipitation of A and B, then we may define an apparent solubility product $K'_{sp} = [A]^\alpha \times [B]^\beta$. The steep ascent of the experimental riptograms for the greater part of their height is their most striking feature, and in this they are very similar to ideal riptograms constructed according to the solubility-product relationship,[7] when β/α is greater than 4. The plateau of the ideal riptograms is seen to have zero slope, in contrast with our experimental riptograms, where we have noted already that another mechanism is removing Orange II from solution.

If the equation for the apparent solubility product is put into the form

$$\frac{\log K_{sp}}{\alpha} - \frac{\beta}{\alpha} \log [B] = \log [A]$$

it should be possible to evaluate β/α graphically from a plot of log [lysozyme] *vs.* log [Orange II]. Carrying out this evaluation for the experiments of Fig. 7 we find the value 11.2 ± 2.7, compared with 13.2 as the mean of four riptograms. Clearly this would not be a very precise way of determining the stoichiometry, since the shape of a riptogram changes very slightly with large changes in β/α, when β/α is larger than about 4. What the calculation does show is that the shape of the experimental riptograms is consistent with the shape calculated from solubility-product assumptions for a precipitate of this composition.

There is a slight curvature apparent at the base of our experimental riptograms, which should, according to the solubility product model, be discontinuous in slope at the base line. The small shifts of solubility of the freshly-formed lysozyme–Orange II precipitate with time (Fig. 2) are of the right sign and magnitude to account for this, at least in part. This small effect may also be due to a small percentage of denaturation or impurity in the lysozyme.

If we were to construct a riptogram according to a Langmuir or Freundlich adsorption equation, we should find that these curves both show a continuous change of slope as they rise from the base-line. In a study of the binding of methyl orange anions by lysozyme[26] Colvin to a considerable extent bases his interpretations on the fact that he obtains rip-

(23) W. A. Ames, *J. Sci. Food Agric.*, **3**, 454, 579 (1952).

(24) C. Tanford and J. Epstein, THIS JOURNAL, **76**, 2163 (1954).

(25) A. Veis and J. Cohen, *ibid.*, **76**, 2476 (1954).

(26) J. R. Colvin, *Canad. J. Biochem. Physiol.*, **32**, 109 (1954).

tograms which leave the base-line tangentially. Since this is a small effect in comparison to the total dye-uptake and may be an artifact, we prefer to regard this region of the riptograms with caution and base our interpretations rather on the prominent features of these curves. As to the general form of the riptograms and in the finding that the dye–protein precipitate has an essentially constant composition during the mutual precipitation, our results confirm those of Colvin.

The very interesting work of Lewin[27] in the preparation of crystalline dye–protein salts suggests that this class of compounds does not necessarily follow stoichiometric relations and may even be thought of as solid solutions. A solid solution should give a linear plot of $\bar{v}$ *vs.* C. Several such tests of the "plateau" data from different lysozyme–Orange II riptograms have given curvilinear plots, tending asymptotically to the maximum value, m. The results of this present work are not in contradiction to those of Lewin but rather show that under adequately defined conditions, the composition of dye–protein precipitates can be definite and constant throughout the whole course of the mutual precipitation.

We may enquire what effect soluble-complex formation between the dye and the protein would have on a riptogram. Since this would result in soluble bound dye passing through the filter and being included in the colorimetric analysis for unbound dye, the situation is analogous to that of incomplete filtration. Further, when sufficient dye has been added to solution to form an insoluble protein–dye precipitate, the *apparent* dye concentration decreases due to the conversion of the soluble complex into precipitate. The over-all effect on a riptogram is that it assumes an S-shape, having a negative slope as it leaves the base-line.[21] Since we observed no indications of an S-shape in our riptograms, we can exclude the existence of soluble lysozyme–Orange II complexes in the systems we have studied. At the highest concentration of lysozyme studied (2×10^{-5} m, pH 3.2, $\Gamma/2$ 0.10, 2.0°), we could have detected such an effect with certainty when a minimum of about 10% of the apparent absorbance was due to a soluble complex. This would mean a free Orange II concentration of 1.8×10^{-5} m and a concentration of bound (soluble) dye one-tenth as great. From this information we can calculate a free energy of association for this hypothetical soluble protein–dye complex of 4.5 kcal. mole^{-1}. Thus if soluble lysozyme–Orange II complexes do exist under the above conditions, their free energy of formation must be less than 4.5 kcal. mole^{-1}.

The system for riptographic analysis which we describe here appears to offer attractive possibilities for the analysis of simple protein mixtures. Preliminary experiments in the titration of insulin, protamine and lactoglobulin and certain mixtures of these proteins with Orange II give some promise of realizing such an analytical goal.

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(27) L. Lewin, THIS JOURNAL, **73**, 3906 (1951).

[CONTRIBUTION FROM THE DEPARTMENT OF CHEMISTRY, UNIVERSITY OF ROCHESTER]

Calabash Curare of the Piaroa Indians. Conversion of C-Curarine-I to C-Curarine-III[1,2]

BY A. ZÜRCHER, O. CEDER AND V. BOEKELHEIDE

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Calabash curare, obtained from the Piaroa Indians, has been studied to determine its chemical nature. Although the mixture is a very complex one, the principal alkaloids have been identified as C-curarine-I, C-toxiferine-II (C-calebassine), C-curarine-III, C-dihydrotoxiferine, caracurine-II and C-fluocurinine. A study of the halochrome reaction of C-curarine-I has led to the discovery that C-curarine-I is converted by acid to C-curarine-III.

Through the kindness of Dr. William J. Robbins of the New York Botanical Garden, a quantity of calabash curare from the Piaroa Indians living in the region of the upper Orinoco in Venzuela has been made available to us for chemical study.[3]

Since all of the curare was gathered at one time from one tribe of Indians, it was anticipated that the various calabashes would be reasonably uniform in alkaloidal content. Physiological tests of crude extracts from various calabashes chosen at random confirmed the fact that the crude extracts from different calabashes were of similar, and high, potency.[4] Likewise, paper chromatograms of random samples showed relatively little variation in content of the principal alkaloids.

Although methods of isolation have improved considerably since Heinrich Wieland's first studies on calabash curare,[5-8] the separation of these complex mixtures into their pure individual components in usable quantities still remains the chief stumbling block to chemical studies in this field. In recent years Karrer, Schmid and their group in

(1) This investigation was supported by a research grant (B-671) from The National Institute of Neurological Diseases and Blindness of the National Institutes of Health, Public Health Service.

(2) Preliminary disclosure of the present findings was made at the International Symposium on Curare, Rio de Janeiro, Brazil, August 4–11, 1957.

(3) Dr. Basset Maguire of the New York Botanical Garden, who has observed the Piaroa Indians during the gathering of plant material and the preparation of the curare, has identified the bark of *Strychnos toxifera*, R. Schomburgh, as being the only important botanical species used.

(4) We are indebted to Dr. Irwin Slater of Eli Lilly and Co. for the pharmacological testing.

(5) H. Wieland, W. Konz and R. Sonderhoff, *Ann.*, **527**, 160 (1937).

(6) H. Wieland and H. J. Pistor, *ibid.*, **536**, 68 (1938).

(7) H. Wieland, H. J. Pistor and K. Bähr, *ibid.*, **547**, 140 (1941).

(8) H. Wieland, K. Bähr and B. Witkop, *ibid.*, **547**, 156 (1941).