# THE HEAT COAGULATION OF HUMAN SERUM ALBUMIN

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The present studies were designed to develop a rapid micromethod for investigation of the effects of heat on relatively concentrated solutions of human serum albumin. The method so devised is proposed for the routine study of commercial preparations, and for inquiry into some of the factors that appear to determine the thermal stability of an albumin solution. As will be clear from the paragraphs that follow, we are using the word stability in a very restricted sense: the studies pertain only to the formation of coagula or aggregates of presumably denatured albumin that are capable of producing a considerable Tyndall effect. The observed phenomenon, which will be referred to as cloud formation, is, according to the views of various authorities (1–10), the net result of two consecutive reactions: the denaturation or unfolding of the protein molecules in solution, and the flocculation, aggregation, or polymerization of the unfolded molecules to form particles that are large enough and present in sufficient concentration to scatter light and give the appearace of a cloud or turbidity. The factors that influence the rate of formation of soluble denatured protein at higher temperatures are not considered in the present study, although work is in progress on a method to ascertain the state of the protein in solution. Likewise, the formation of soluble cleavage products that might arise from thermal degradation under special conditions is not considered.

## *Method*

The method now employed is based upon the use of thin walled capillary tubes, and the heating of albumin solutions contained therein at a constant and sufficiently high temperature until a cloud forms in the solution; that is, until the cloud point[1] is reached. Under optimum conditions the rate of cloud formation is quite rapid, and the time required for attainment of a 30 second cloud point may be recorded to within a few seconds.

Before the present studies were undertaken, Dr. Paul Tompkins of this

[1] The "cloud point" is apparently closely related to and perhaps synonymous with the coagulation point. It is so described because, under the conditions of these experiments, the light-scattering phase appears to the observer, at the moment of recording, as a haze or cloud rather than as a heavy coagulum.

laboratory made substantial progress with a method that called for elevation of the bath temperature at a linear rate, from an initial level of 50° or higher to the cloud point temperature; the observer recorded both the cloud point temperature and the cloud point time. Either value alone or the product of the two was found to provide descriptive information which was of qualitative interest but the data were not amenable to further analysis or interpretation.

Lepeschkin (3) has described a method that was devised upon a sufficiently small scale with respect to demand for material, but this was technically unsatisfactory for application to the problem at hand. No attempt was made to inquire, by actual trial, into its further possibilities.

*Capillary Tubes*—We have found that the most satisfactory capillaries are of thin walled soft glass, prepared by heating and drawing out ordinary 6 inch test-tubes, and which have about the following dimensions: length 8 cm. and external diameter 1.5 mm. To indicate the thickness of wall that is considered desirable we find that ten of our tubes selected at random weigh 0.70 gm., or 70 mg. each. Commercially available melting point tubes were tried but their use was discontinued owing to their insufficient length and their larger and variable diameter. The thicker walls and greater diameters of the melting point tubes do not permit a sufficiently rapid equilibration of temperature. An increase in inside diameter of from 0.7 to 1.5 mm. increased the cloud point time in a given experiment from 26 to 35 seconds, *i.e.* by 9 seconds, and a change from one of our thin walled capillaries to a melting point tube of the same internal diameter raised the cloud point time from 33 to 36 seconds. However, melting point tubes of constant diameter could be used with fair accuracy for comparative purposes.

*Filling*—The protein solution is drawn about one-third of the way up the tube by gentle suction, the empty end is sealed in a small flame, and the contents are transferred to the sealed end after it cools by shaking with a whipping motion of the hand.

*Water Bath*—For a constant temperature bath we have found that one of brass plate (1.3 mm. thick) containing three windows of plate glass is satisfactory. The internal dimensions of the bath are as follows: length 9 inches, width 6 inches, and depth 10 inches. A box of $\frac{3}{4}$ inch, five-ply wood was built to fit snugly over the outside of the brass plate bath, and to furnish a moderate amount of thermal insulation. Greater insulation of the sides of the bath is in all probability unnecessary because the top of the bath is uncovered.

The diagrams in Fig. 1 illustrate the arrangement of the three windows in the bath, the position of the light projector, and the location of the temperature-regulating equipment. A concentrated beam of light from a micro projector is passed through the bath and out through a window on the

opposite side in order to avoid reflection from the bath walls. To serve as a background a piece of black bakelite, fastened to a strip of brass for support on the sides of the bath, extends down into the water about 5 inches, that is to a point just below the bottom of the windows, and thus does not interfere with the circulation of the bath water. The light that is scattered by the protein solution in the immersed capillary is observed through the front window against the bakelite background, preferably in a darkened room. Mueller (11) has reported that the intensity of scattered light is maximal at an angle of 135° to the incident beam. We have found that the cloud point is definitely more distinct when observed at an angle to the incident beam somewhat greater or less than 90°.

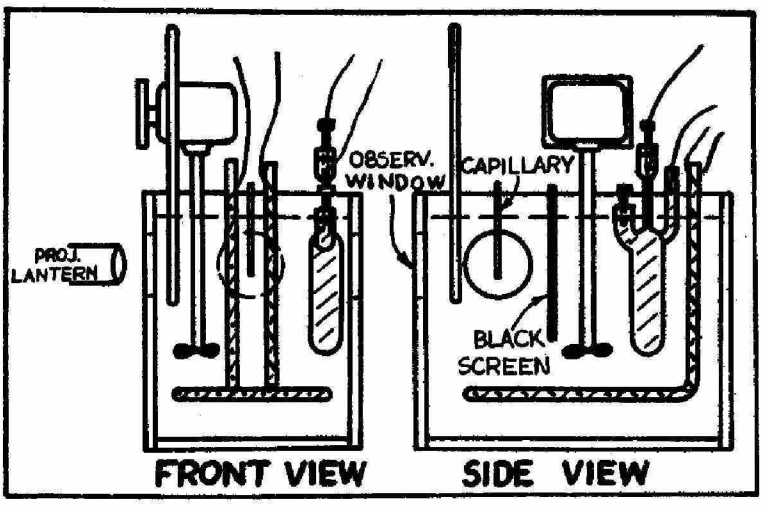


FIG. 1. Constant temperature bath

The bath is refilled with distilled water every 2 or 3 days in order to keep the water clear and free from dust. The heating element employed is an 8 foot length of chromel resistance wire, wound in a $\frac{1}{2}$ inch spiral, and supported in the bath with a Pyrex rod or tubing of small diameter passed through the spiral. A mercury thermoregulator of conventional design is used, except that it is provided with a side arm into which is inserted a rubber stopper with a set screw. Rapid adjustment of the temperature is facilitated with the screw. A variation of $\pm 0.03°$ in bath temperature is obtained by arranging the relay and heater circuit so that only a part of the current through the element is controlled by the relay.

*Technique*—In carrying out a determination it is advisable to have the solution in the capillary only half immersed, for if the filled portion is completely immersed, cloud formation will begin at the air-liquid interface and work downwards, thus decreasing the sharpness of the cloud point, and perhaps even shortening the time for its appearance. Another advantage may also be realized from partial immersion of the protein solution; if the

observer, shortly before the appearance of the cloud, lowers the capillary another 0.5 to 1.0 cm. in the bath, he will have a small amount of the unclouded solution to compare with the clouding portion, and by contrast such action will tend to sharpen the observed cloud point. The temperature of the bath should be so adjusted as to give a cloud point appearance time of 10 to 60 seconds. If the cloud point time is less than 5 seconds, we assume the value to be of doubtful significance, since an appreciable proportion of the time would probably be required for equilibration of temperature, the capillary being at room temperature prior to immersion. If the cloud point time is greater than 150 seconds, difficulties are encountered owing to loss of sharpness of the cloud point.

## EXPERIMENTAL

Throughout these studies use has been made exclusively of crystalline human serum albumin or of amorphous serum albumin which, on electrophoretic analysis, appeared to contain not more than 2 per cent of globulin (mostly $\alpha$-). These preparations have recently been characterized in several papers (12–16). Unless otherwise stated, 25 gm. per cent solutions of the protein were used. The solutions were prepared by mixing in the dry state albumin and sodium carbonate, the latter in quantities sufficient to give a reaction close to the desired pH. Water was added and solution of the albumin effected at room temperature or at 0°. A stock solution, 33.3 per cent albumin, was thus prepared. 75 cc. portions of these stock solutions were diluted with an appropriate volume of sodium chloride of the desired molarity. In certain of the experiments to be reported, in which other salts were studied, dilution was made with the required volume of solutions of these other substances. The final volumes after the additions mentioned were 100 cc., thus giving solutions that contained 25 per cent albumin. Suitable allowances were made for the water contained in the "dry" albumin preparations used. The investigation pertains, therefore, to 25 gm. per cent solutions of albumin, computed as for anhydrous material.

Before determinations of pH were made, the concentrated albumin solutions were diluted to 25 volumes with water. Although this procedure does not give the pH of the 25 per cent solutions actually employed, measurements were made in this way in conformity with the conventions now employed in all laboratories in which work with these solutions of human serum albumin is being done. It must therefore be emphasized that wherever reference is made in the present paper to the pH of an albumin solution we mean the pH of a solution which has been diluted to contain about 1 per cent of protein.

Determinations of the cloud point are made at several temperatures within a range so chosen as to give a series of cloud point times of from 10 to 60 seconds. The significance of this procedure will appear later.

*Studies on Typical Preparations*—Determinations of the cloud point time (C. P.) have been made thus far on thirty-six different preparations[2] at several different temperatures. A semilogarithmic plot of the data so obtained, log C. P. against temperature, gave rise to a family of straight lines such as are illustrated in Fig. 2 for ten of the preparations selected at random. The slopes of these lines are very nearly the same. Later studies indicate that the slope is determined in part by the protein concentration, the nature of the added salt, and also by the pH. The linear relationship that we have observed between the logarithm of the cloud point time and the temperature is in confirmation of the findings of Buglia (17), whose

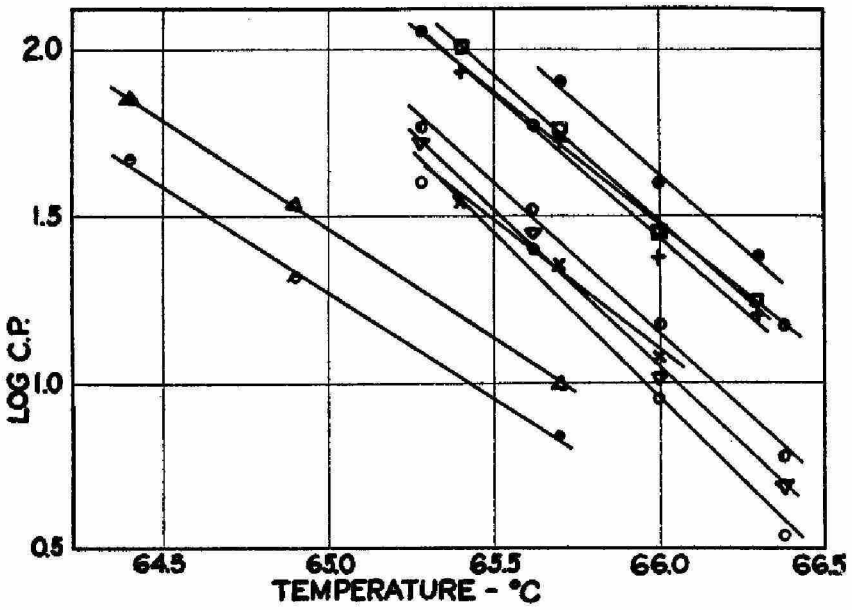


FIG. 2. Cloud point-temperature relationship. 1:25 dilution. pH ◓ 7.15, ○ 7.01, ◐ 6.81, ◑ 6.70, ● 6.55, △ 7.17, ▽ 6.80, □ 6.56, × 6.50, + 6.52.

work was carried out under quite different conditions but, none the less, was such as to demonstrate the relationship mentioned.

The results obtained on thirty-six different amorphous preparations are presented in Table I. This serves to indicate the spread of values encountered in ordinary albumin processing. It also reflects the quality of the albumin, since all preparations of low cloud point are found to be poor as judged by nephelometric study in the course of standard stability tests at 57°.

In Table I it will be noted that the entries in the second and third columns

[2] The preparations used for this purpose were the products of an industrial house engaged in the large scale preparation of human serum albumin. As received by us, they were already in 25 per cent solution, contained 0.3 M sodium chloride, and were of pH 6.42 to 7.28, with most values clustered around pH 6.8. The sodium carbonate additions, made at the time of dissolving the albumin, were such as to add about 0.05 to the ionic strength.

are described, respectively, as "Temperature for 30 second C. P." and "Calculated C. P. at 65°." These values were obtained in all cases by making a few determinations of C. P. within the appropriate temperature range. A linear plot, log C. P. *versus* temperature, was constructed from these observations and from this in turn the values presented in the second and third columns of Table I are readily calculated.

Quadruplicate determinations of the cloud point are made for any given temperature upon each preparation, and the average C. P. for the contents

TABLE I

*Routine Cloud Points*

| Preparation No. | Temperature for 30 sec. C. P. | Calculated C. P. at 65° | Preparation No. | Temperature for 30 sec. C. P. | Calculated C. P. at 65° |
|---|---|---|---|---|---|
| | °C. | *sec.* | | °C. | *sec.* |
| 26 | 65.73 | 117 | 46 | 66.09 | 230 |
| 27 | 66.28 | 327 | 47 | 65.27 | 58 |
| 28 | 65.59 | 90 | 48 | 66.14 | 252 |
| 31 | 66.03 | 201 | 49 | 66.18 | 272 |
| 32 | 66.16 | 262 | 50 | 66.32 | 352 |
| 33 | 66.11 | 238 | 51 | 66.48 | 475 |
| 34 | 66.11 | 238 | 52 | 66.21 | 287 |
| 35 | 65.98 | 187 | 53 | 66.34 | 366 |
| 36 | 65.91 | 168 | 54 | 66.29 | 318 |
| 37 | 65.96 | 180 | 55 | 66.16 | 262 |
| 38 | 65.89 | 162 | 56 | 65.55 | 84 |
| 39 | 66.27 | 221 | 57 | 66.30 | 340 |
| 40 | 66.18 | 272 | 58 | 66.14 | 252 |
| 41 | 65.83 | 141 | 59 | 66.48 | 475 |
| 42 | 66.25 | 309 | 60 | 66.64 | 641 |
| 43 | 66.16 | 262 | 61 | 66.46 | 457 |
| 44 | 65.79 | 131 | 90X | 64.25 | 7 |
| 45 | 66.04 | 204 | 22 | 65.01 | 31 |

of the four capillaries is recorded. In the 30 second range such quadruplicates should agree to ±1 second.

It is probable that the semilogarithmic plot is linear for only a comparatively narrow temperature range. This is suggested by the observation that if the data now at hand were to be recalculated for a cloud point temperature of 50° the C. P. of all preparations studied of late would be many million years, a conclusion which is not in harmony with actual observations on the stability of these preparations at 50°.

*Arrhenius Constant*—Values for the activation energy, $E$, or the Arrhenius constant were calculated with the aid of the following expression,

$$\log \frac{(\text{C. P.})_{t_1}}{(\text{C. P.})_{t_2}} = \frac{E}{2.3R}\left(\frac{T_2 - T_1}{T_m{}^2}\right)$$

TABLE II

*Arrhenius Constant*

| Effect of variations in | pH (1:25) | $-\frac{\text{Log C. P.}}{t}$ | $T_m$ | $E \times 10^{-3}$ | |
|---|---|---|---|---|---|
| pH; calculated from data in Fig. 3 | 5.1 | 0.29 | 273 + 63 | 150 | |
| | 5.6 | 0.46 | + 66 | 240 | |
| | 6.4 | 0.58 | + 67 | 310 | |
| | 7.9 | 0.55 | + 66 | 290 | |
| | 8.5 | 0.4 | + 65 | 210 | |
| | 9.5 | 0.36 | + 66 | 190 | |
| | 10.0 | 0.37 | + 66 | 200 | |
| | 10.7 | 0.18 | + 66 | 95 | |
| | NaCl | | | | |
| | *M* | | | | |
| NaCl concentration; from data in Fig. 6 | 0 | 0.60 | 273 + 63 | 310 | |
| | 0.15 | 0.64 | + 66 | 340 | |
| | 0.30 | 0.60 | + 67 | 320 | |
| | 0.60 | 0.62 | + 69 | 330 | |
| | 0.90 | 0.74 | + 71 | 400 | |
| | 1.50 | 0.62 | + 72 | 340 | |
| | Protein concentration | | | | |
| | *per cent* | | | | |
| Protein concentration; from data in Fig. 5 | 5 | 0.26 | 273 + 72 | 140 | |
| | 25 | 0.60 | + 67 | 320 | |
| | 45 | 0.80 | + 66 | 420 | |
| | 0.3 M | | | | pH |
| Nature of added substance | Blank | 0.60 | 273 + 63 | 310 | 6.73 |
| | Chloride | 0.62 | + 67 | 320 | 6.78 |
| | Acetate | 0.61 | + 68 | 330 | 6.88 |
| | Propionate | 0.80 | + 71 | 430 | 6.88 |
| | Butyrate | 0.74 | + 74 | 410 | 7.05 |
| | Valerate | 1.06 | + 79 | 610 | 6.75 |
| | Caproate | 0.56 | + 80 | 320 | 6.78 |
| | Succinate | 0.58 | + 67 | 310 | 7.03 |
| | Fumarate | 0.60 | + 68 | 320 | 6.92 |
| | Lactate | 0.69 | + 69 | 370 | 6.81 |
| | Glucose | 0.36 | + 65 | 190 | 6.75 |

where $T_m$ is the absolute temperature of about the middle of the measurable cloud point range, which is seldom wider than 3°. It was assumed that $(\text{C. P.})_{t_1}/(\text{C. P.})_{t_2} = (k_2)/(k_1)$, where $k_2$ and $k_1$ are the reaction rate constants

for the over-all reactions which lead to the observed C. P. at temperatures $t_2$ and $t_1$ (see also (18)). For the present no attempt will be made to discuss the significance of the different values of $E$ obtained.

Table II presents values of $E$ with variation of pH, sodium chloride concentration, or protein concentration, and for a series of different added substances. Crystalline albumin was used to obtain the data presented in Table II.

*Effect of pH*—The optimum pH for the high temperature thermal stability of human serum albumin in 25 per cent solution and 0.3 M in sodium chloride was determined on two preparations. The results obtained

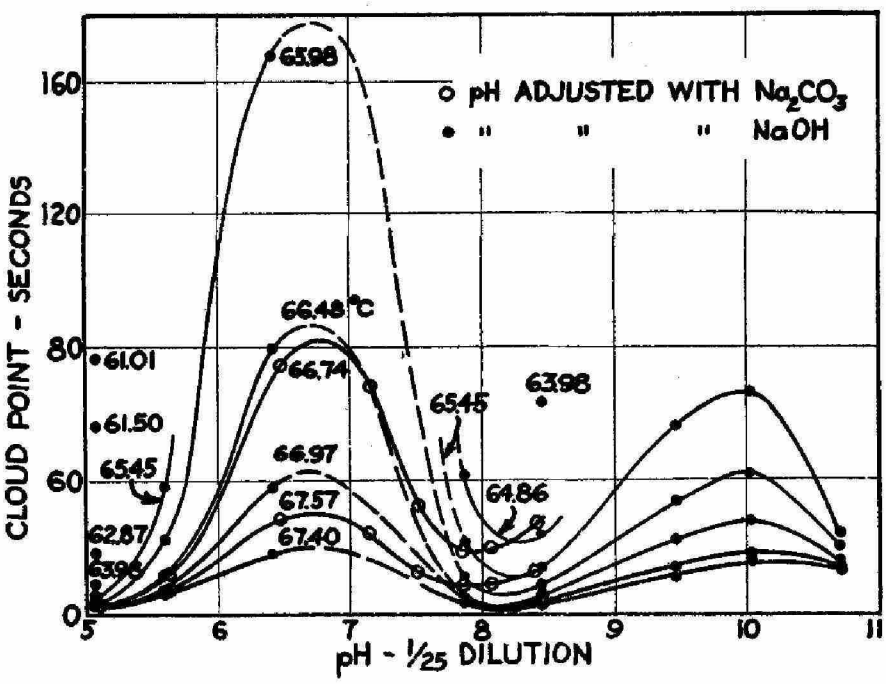


FIG. 3. Effect of pH on cloud point

on one of these preparations (crystalline) are presented graphically in Fig. 3. The appearance of a second optimum at pH 10 should be noted as well as the relationship between pH and $\Delta$ log C. P. $/\Delta t$; this relationship is illustrated further in Fig. 4 and in Table II. The determination of the optimum pH as 6.8 was first reported by Scatchard *et al.* (13) by the use of viscometric and nephelometric methods.

*Effect of Protein Concentration*—An investigation was made of the effect of variations in protein concentration at constant total salt concentration (0.3 M) and constant pH, upon the cloud point temperature. The results, presented in Fig. 5, seem to indicate that the thermal stability varies inversely with the protein concentration.

Determinations of cloud point were made at several temperatures for both the 5 and 45 gm. per cent albumin solutions in order to permit plot-

ting log C. P. against temperature. The slopes of the straight lines so obtained were −0.26 and −0.80 instead of −0.63, as was regularly obtained with this crystalline albumin preparation in 25 gm. per cent solution. It appears to follow that the value of $a$ (slope) in the equation log C. P. = $at + b$ is, in part, a function of albumin concentration.

It could also be computed that this 9-fold increase in protein concentration (5 to 45 per cent) decreased the 65° C. P. about 35-fold.

It is interesting to note that the increase in the slopes mentioned above in going from a concentration of 5 to 45 per cent protein corresponds to an increase in the Arrhenius constant (see Table II) of from 140,000 to 420,000 calories. However, in consideration of the decrease in C. P. with increase in protein concentration, it would seem that the "thermal sta-

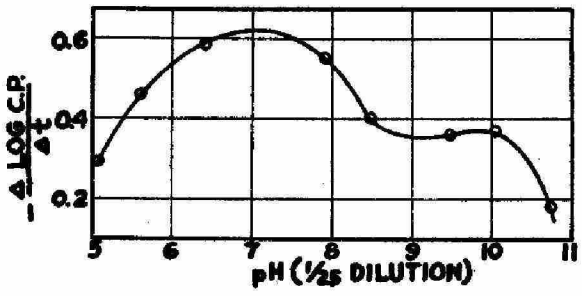


FIG. 4

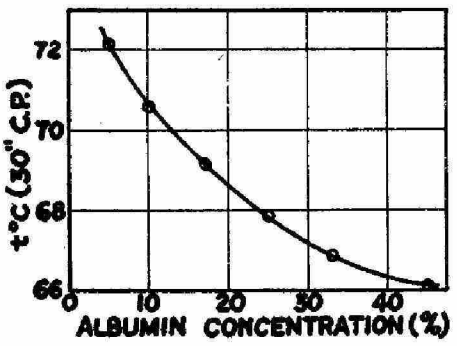


FIG. 5

FIG. 4. Effect of pH on Δ log C. P./Δ$t$.
FIG. 5. Effect of albumin concentration on cloud point.

bility," as defined in this work, decreases with increase of protein concentration. It is quite probable that the reaction rate of aggregation of the denatured albumin to form light-scattering material is dependent on the concentration of the denatured albumin.

*Effect of Ionic Strength*—The effect of variations in the ionic strength (sodium chloride used) at constant protein concentration (25 per cent) and constant pH was also determined. These results are presented in Fig. 6; they confirm essentially the findings reported by Scatchard *et al.* (13). Incidentally it was observed that the slope of the log C. P.-temperature curve did not vary with change of sodium chloride concentration.

*Effect of Constant Mole Ratio (NaCl to Protein)*—Since increase of ionic strength in a system containing 25 per cent albumin is sufficient to increase the cloud point markedly (Fig. 6), it was suggested that the results presented in Fig. 5 might be due to the high mole ratio (NaCl to protein) that prevailed in solutions of low protein content. In consequence the experiments illustrated by Fig. 7 were conducted. In these experiments a constant mole ratio for salt and protein was maintained. It is evident from

Curve II (sodium chloride) that the increase of cloud point with decrease of protein concentration (Fig. 5) is not due to increase of the mole ratio (NaCl to protein) in systems of low protein content. However, the results do not permit of a very simple explanation, as is indicated by the behavior of sodium butyrate (Curve I, Fig. 7).

*Specific Anion Effects*—In a preliminary study, replacement of part of the sodium chloride, routinely employed, with sodium acetate was investigated. With the two preparations studied there was a marked increase in the 30 second cloud point temperature. With these findings before us, a systematic study was made of other sodium salts added to a solution of

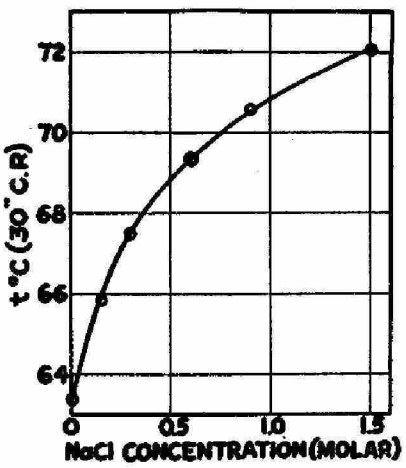


FIG. 6

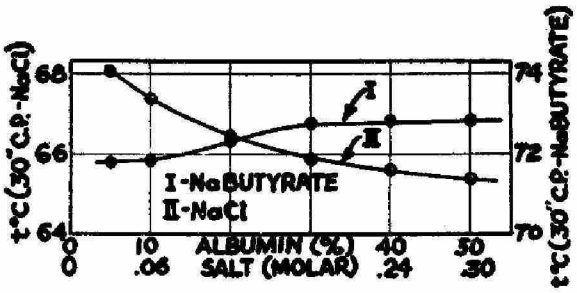


FIG. 7

FIG. 6. Effect of NaCl concentration on cloud point.

FIG. 7. Effect on 30 second cloud point temperature of the simultaneous variation of the albumin and salt concentrations.

crystalline albumin. The results are presented in Table III. The increase in cloud point with increase in length of the fatty acid anion is striking. Sodium caprylate is the highest member of the homologous series that we have studied. In 0.3 M caprylate a 25 per cent solution of serum albumin failed to show cloud formation even at 100°. It was noted, however, that the solution set to a clear translucent gel at temperatures between 80–100°.

A few experiments were performed on two routine amorphous preparations (Nos. 47 and 51). The results are presented in Table IV. The absolute values for comparable experiments, reported upon in Tables III and IV, disagree because of the relatively high thermal stability of crystalline preparations. Qualitatively, however, there is good agreement.

*Other Added Substances*—In Table III we have included results with substances other than sodium salts. Alanylglycine was investigated with the thought in mind that substances of high dipole moment might enhance the thermal stability. Negative results were encountered with this substance. $\alpha$-Globulin (about 85 per cent $\alpha$-globulin on electrophoretic

analysis) was studied because it is the commonest protein impurity in serum albumin preparations. It is significant that this material did not decrease the high temperature thermal stability.

TABLE III

*Increase of Thermal Stability of Serum Albumin As Affected by Added Substances*

| Substance added | Concentration | pH | 30 sec. C. P. temperature |
|---|---|---|---|
| | *M* | | °C. |
| Sodium succinate | 0.3 | 7.03 | 66.62 |
| " chloride | 0.3 | 6.78 | 67.29 |
| " fumarate | 0.3 | 6.92 | 68.35 |
| " lactate | 0.3 | 6.81 | 69.22 |
| " phenyl acetate* | 0.3 | 6.76 | 78.20 |
| " acetate | 0.3 | 6.88 | 68.02 |
| " propionate | 0.3 | 6.88 | 71.48 |
| " butyrate | 0.3 | 7.05 | 74.07 |
| " butyrate* | 0.3 | 6.97 | 75.09 |
| " valerate* | 0.3 | 6.75 | 78.40 |
| " caproate* | 0.3 | 6.78 | 79.96 |
| " caprylate* | 0.3 | Did not coagulate at 100° | |
| " chloride* | 0.3 | 6.64 | 67.44 |
| Glucose* | 0.3 | 6.75 | 65.12 |
| Sodium chloride† | 0.3 | 6.77 | 67.87 |
| Alanylglycine† | 0.3 | 6.62 | 66.27 |
| Sodium glycerophosphate† | 0.3 | 7.49 | 64.68 |
| " chloride + α-globulin† | 0.3<br>1.7% | 6.91 | 68.05 |
| " chloride‡ | 0.15 | 7.28 | 63.1 |
| " " | 0.3 | 7.06 | 65.4 |
| " " | 0.15 | 7.28 | 71.7 |
| " butyrate | 0.15 | | |
| " chloride | 0.15 | 6.84 | 76.9 |
| " phenyl acetate‡ | 0.15 | | |
| " chloride | 0.15 | 7.15 | 81.5 |
| " phenyl butyrate‡ | 0.15 | | |
| " chloride | 0.15 | 6.68 | 81.8 |
| " caprylate‡ | 0.15 | | |

* Second series of experiments.

† Third series of experiments.

‡ Fourth series of experiments; amorphous preparation used.

*Nephelometry at 50° and 57°*—In order to bring the present results into correlation with the routine tests applied to albumin solutions produced industrially as well as to inquire further into the effects of added salts, parallel nephelometric experiments were conducted at 50° and at 57°. To this end, bottles of appropriate shape containing 15 cc. samples of test solutions of 25 per cent albumin were employed and the rate of development

of the light-scattering phase was studied. Two amorphous preparations, designated Preparations D and 94-95 respectively, were used. Readings

TABLE IV

*Confirmatory Experiments with Routine Preparations*

| Preparation No. | Added salt (0.3 M) | 30 sec. C. P. temperature | 65° C. P. time | Ratio of 65° C. P. times |
|---|---|---|---|---|
| | | °C. | sec. | |
| 47 | Sodium chloride | 64.77 | 19 | 1 |
| 47 | " propionate | 65.72 | 115 | 6 |
| 47 | " butyrate | 67.52 | 2,280 | 120 |
| 51 | " chloride | 66.08 | 225 | 1 |
| 51 | " propionate | 67.27 | 2,060 | 9 |
| 51 | " butyrate | 68.76 | 33,650 | 150 |

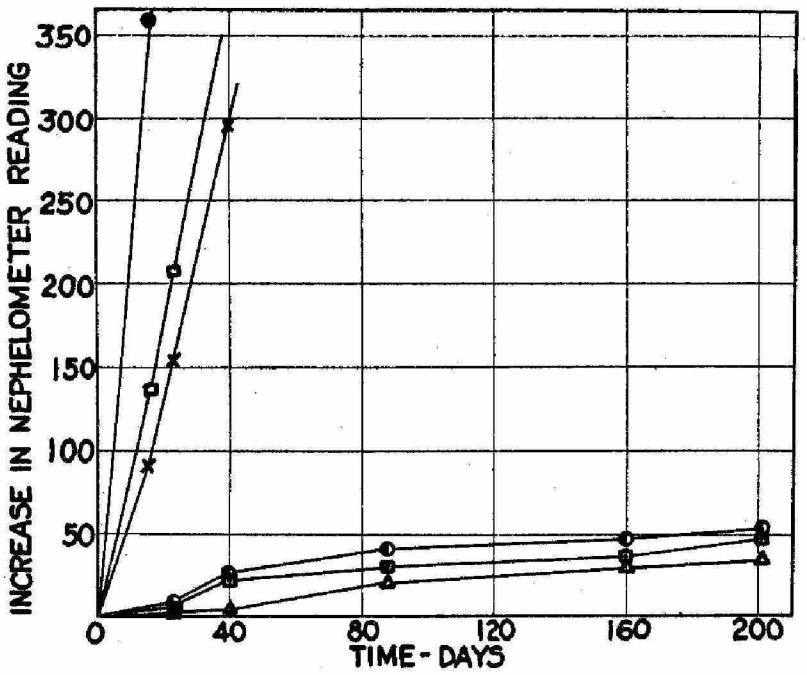


FIG. 8. Effect of various salts on the stability of Cutter Preparation D at 57°. ● 0.15 M chloride, × 0.15 M chloride and 0.15 M chloride, ⊡ 0.15 M chloride and 0.15 M butyrate, △ 0.15 M chloride and 0.15 M phenyl acetate, ◐ 0.15 M chloride and 0.15 M caproate, □ 0.15 M chloride and 0.15 M lactate.

were made in arbitrary units on a Zeiss nephelometer in conjunction with a Pulfrich photometer. The results are illustrated in Figs. 8 to 11.[3] The

[3] The ordinates in Figs. 8 to 11 present the increase in nephelometric readings obtained under the conditions of these experiments. An absolute reading of 20 units on the Zeiss nephelometer corresponds to a concentration of light-scattering material such that a 1 inch thickness of solution is barely detectably turbid when examined in bright light with the naked eye. Solutions that read 10 units or so are completely transparent and are optically clear to the naked eye. The solutions used in these studies almost invariably have initial absolute readings of 5 to 10 units.

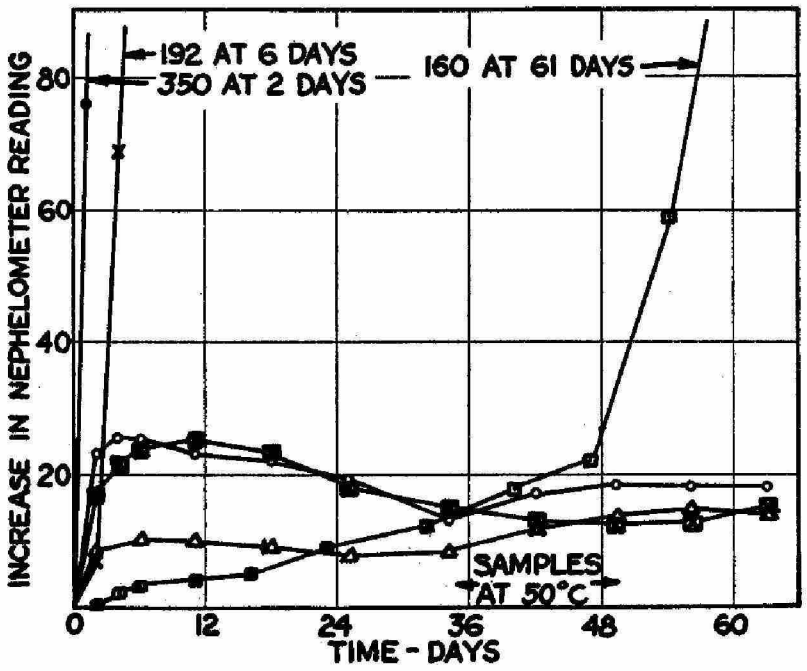


FIG. 9. Effect of various salts on the stability of Preparation 94-95 at 57°. ●, ×, ⊡, △ as in Fig. 8; ⊠ 0.15 M chloride and 0.15 M caprylate, ○ 0.15 M chloride and 0.15 M phenyl butyrate.

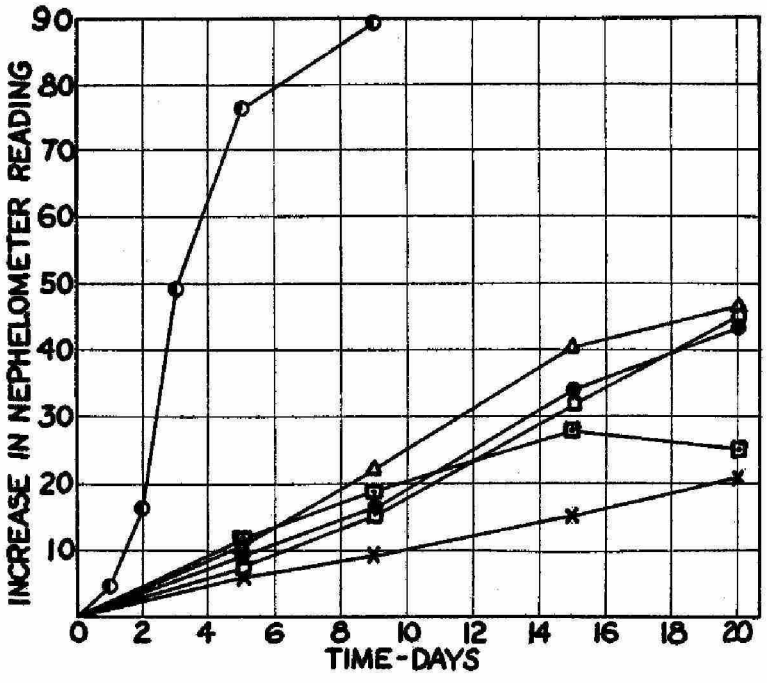


FIG. 10. Effect of various salts on the stability of Cutter Preparation D at 50°. The symbols have the same significance as in Fig. 8.

marked stabilizing effects of butyrate, caprylate, phenyl acetate, and phenyl butyrate at 57° are clearly in evidence.

The instability observed in the 50° experiments with caproate and capry-

late and the comparatively high stability conferred by caprylate at 57° or at cloud point temperatures seem, on superficial examination, to be in conflict. A complete explanation cannot be presented at this time, although the data now at hand permit us to conclude that the concentration of non-polar anion, its denaturing power, the length of the carbon chain of the non-polar anion, and the temperature are the most important factors.

Preparation D, incidentally, is a singularly unstable and rather atypical preparation. It was used only to see whether we could effect stabilization of an admittedly labile product. The other albumin, Preparation 94-95, is

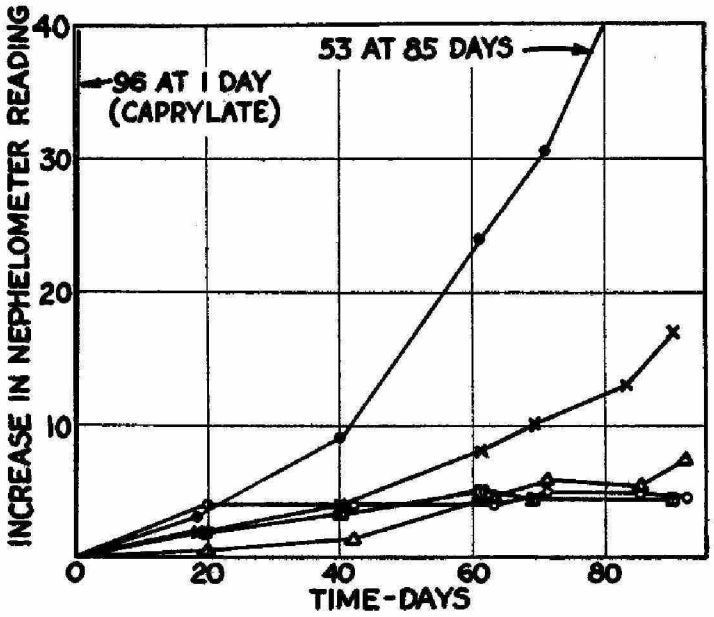


FIG. 11. Effect of various salts on the stability of Preparation 94-95 at 50°. The symbols have the same significance as in Fig. 9.

a typical representative of present day albumin preparations as produced industrially.

## DISCUSSION

The most significant result of the studies reported herein is the increase in cloud point time and temperature of serum albumin solution with increase in chain length of the added fatty acid ion. From these data, however, definite conclusions may not be drawn regarding the effects of the added substances on the two reactions of the protein, denaturation and flocculation. It is presumed that the two reactions are quite separate and that the latter must be preceded by the former; that is, under the conditions of these experiments only denatured protein would be susceptible to aggregation into flocs.

Two possibilities deserve discussion if only to lay an appropriate basis

for further investigation of the phenomenon. (*a*) The added substance may combine with the protein in its native state, giving rise to a product which is not readily susceptible to denaturation by heat and, hence, to flocculation. (*b*) The added substance may not combine to any appreciable degree with the protein in its native state. Following upon heat denaturation, however, combination may ensue with the added substance just as rapidly as opening out of the molecule exposes appropriate points of combination. (These, incidentally, are assumed to be free amino groups and the aliphatic side chains of amino acid residues, especially of those that carry the free amino groups.) Association with the amino groups ($R—\dot{N}H_3^+$) would be through electrostatic attraction with the carboxyl group of the added substance ($R'—COO^-$). Association with the non-polar groups would be through van der Waal's forces which, considered in the aggregate, would increase with increase in length of the carbon chain of the added substance. Either hypothesis necessitates the conclusion that the protein-fatty acid anion complex is of much greater solubility than denatured protein itself. Not excluded from consideration is a type of combination in which association with the protein is through the non-polar portion of the added anion, the carboxyl group being immersed in the aqueous solvent.

A factor that may be involved in the variable influence of the members of the homologous series is that of a varying affinity of the acid anions for the protein. Steinhardt, Fugitt, and Harris (19) have reported an increasing affinity of anions for egg albumin with increasing dimensions of the anion.

At present we are disposed to favor the hypothesis of combination with denatured protein, partly because the high temperature at which cloud formation ensues is, of itself, conducive to denaturation, and partly because the higher fatty acid salts ($C_{12}$ and up) or derivatives thereof possess some degree of detergent power. It is possible that a similar detergent and hence denaturing property may be manifest in some of the lower fatty acid salts such as those that we have used. An increase in temperature, incidentally, appears to enhance the denaturing effect of detergents (20).

The comparatively high solubility of the protein-fatty acid anion complex, or at least its resistance to flocculation, may be attributed to a masking of all free amino groups through combination with the fatty acid anion. Such a combination would leave the protein with a relatively high negative charge as conveyed by the free carboxyl groups of the protein molecule. This, in turn, would militate against coalescence of particles and formation of a coagulum.

Several studies are now in progress which are designed to throw further light on the data presented in this paper: cloud point investigations on

heated protein-fatty acid anion systems before and after dialysis against 0.3 M sodium chloride; quantitative determination of denatured protein in such systems by a papain method; and electrophoretic analysis.

## SUMMARY

1. A "cloud point" method, in which thin walled capillaries are used as containers, is described for studies on the thermal coagulation of proteins.

2. Investigation of many preparations of human serum albumin show that, under the conditions of these experiments, a linear relationship exists between the logarithm of the cloud point time and the corresponding cloud point temperature. This relationship may be expressed by the equation log C. P. $= at + b$. The slope, $a$, is determined in part by the protein concentration, the pH, and by the nature of any salts present, but is independent, within limits, of the concentration of sodium chloride.

3. Cloud point data, expressed as the 30 second cloud point temperature and the 65° cloud point time, are recorded for many commercial preparations of human serum albumin.

4. A low cloud point appears to be referable to changes in the albumin itself and not to contamination with globulins or salts.

5. The optimum pH for the high temperature thermal stability of human serum albumin in 25 per cent solution and in 0.3 M NaCl was found to be 6.6, with a secondary optimum at pH 10.

6. Within the protein concentration range, 5 to 45 per cent, the cloud point was found to vary inversely with the concentration of protein.

7. In confirmation of the observations of others, the thermal stability of serum albumin was found to increase with increase of sodium chloride content.

8. In a system of constant mole ratio, NaCl to protein, the cloud point of serum albumin varies inversely as the protein concentration.

9. If the added electrolyte is a sodium salt of one of the lower fatty acids instead of sodium chloride, the cloud point increases with ascent of the homologous series; *i.e.*, with increase in length of the carbon chain. The protective effect of phenyl acetate is as great as that of valerate, while phenyl butyrate is about as effective as caprylate. These conclusions apply to systems containing the fatty acid in the concentration of 0.15 to 0.3 M.

10. Nephelometric studies at 57° confirm, qualitatively, the results of cloud point studies in the 65–75° range. At 50° there is also qualitative agreement except in the case of caproate and caprylate.

The work described in this paper was done under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

Dr. John Edsall of the Department of Physical Chemistry, Harvard Medical School, proposed the experiment with propionate, which led, in turn, to investigation of the salts of higher fatty acids. Much of the serum albumin and many other proteins required for this work were provided by the Harvard Plasma Fractionation Laboratory through the courtesy of Professor E. J. Cohn. We are indebted to the Cutter Laboratories for much cordial cooperation and to Professor Linus Pauling for assistance in interpreting some of the findings that came to light in the present study.

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# THE HEAT COAGULATION OF HUMAN SERUM ALBUMIN

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