

CHEMICAL, CLINICAL, AND IMMUNOLOGICAL STUDIES ON THE PRODUCTS OF HUMAN PLASMA FRACTIONATION.

V. THE INFLUENCE OF NON-POLAR ANIONS ON THE THERMAL STABILITY OF SERUM ALBUMIN^{1,2,3}

By GERALD A. BALLOU, PAUL D. BOYER, J. MURRAY LUCK,
AND FUNSTON G. LUM

(From the Biochemical Laboratory, Department of Chemistry, Stanford University, California)

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The present studies were initiated to increase still further the high stability that is characteristic of normal human serum albumin as prepared for the armed forces (1). The nephelometric and viscosimetric characterization of the standard solution has been discussed in detail in the preceding paper of this series (2).

There is the well-known solubilizing action on certain proteins of non-polar anions of comparatively high molecular weight. Some of these are the higher fatty acid anions; others are mixed aliphatic-aromatic anions of sulphonic acid. The latter are in common use as detergents. They not only solubilize many proteins but effect denaturation as well. The high temperature stability of serum albumin, however, was observed to be appreciably increased by the presence, in moderate concentration, of acetate ion. From this, it was considered that higher fatty acid anions would be deserving of study.

All of this work has been restricted to comparatively concentrated solutions of human serum albumin. The systems investigated are those described and characterized in the first, second, and fourth papers in this series (1 to 3).

¹ This work has been carried out under contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Stanford University.

² The products of plasma fractionation employed in this work were developed from blood, collected by the American Red Cross, by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract, recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University.

³ We are indebted to Dr. John Edsall for suggesting experiments with propionate, to Professor Linus Pauling for assistance in the interpretation of some of the findings in the present study, and to the Cutter Laboratories for much cordial cooperation.

The conclusions drawn from the observations to be reported are not necessarily applicable to solutions of lower protein concentration.

The observations, furthermore, have been restricted to thermal stability at 50°, 57°, and at "cloud-point" temperatures. In the first 2 cases, we have applied conventional nephelometric technics with the modifications described in the fourth paper of this series (2), and designed to permit quantitative study of the formation of coagula or aggregates capable of producing a measurable change in the Tyndall effect. As has been pointed out (2), the method is sensitive; only a very small proportion of the total protein present contributes to the light scattering. The "cloud-point" method is also based on light scattering, but the proportion of total protein that participates in light scattering is higher than in the nephelometric method. Expressed differently, we may say that the end-points for the two methods are at very different levels.

METHOD

The nephelometric studies (50° and 57°) were carried out in the 15 cc. vials designed⁴ for use in the Zeiss Nephelometer which is used in conjunction with the Pulfrich Photometer.

The cloud-point studies were carried out in thin-walled capillary tubes. The albumin solutions contained therein were heated at a constant and comparatively high temperature until a sharply discernible cloud formed in the solution,—that is until the cloud-point was reached. The body of coagulated protein forms as a haze or cloud rather than as a heavy coagulum, under the conditions of these experiments. If the conditions are optimum, such that the cloud-point time is in the 10 to 60 second range, quadruplicate specimens agree to within 2 or 3 seconds. In

⁴ Lieutenant Commanders L. M. Woodruff and S. T. Gibson, Medical Corps, United States Naval Reserve, are responsible for the design of these vials.

most cases, the cloud of coagulated protein disappears at once if the tube be withdrawn from the bath within a second or two of attaining the end-point. Within limits, cloud formation is repeatedly reversible on repeated heating and cooling though the cloud-point time becomes progressively shorter. In application of the method, use is made of a small constant temperature bath with windows, a projector for illumination of the capillary tubes, and thermoregulation to $\pm 0.03^\circ \text{C}$. A fully detailed description of the cloud-point technic, suitably illustrated, has been published elsewhere (6).

EXPERIMENTAL

We have used exclusively either crystallized human serum albumin or standard serum albumin, characterized in other papers of this series (1 to 5). Solutions were prepared by mixing in the dry state albumin and sodium carbonate, the latter in quantities sufficient to give almost the desired pH. Water was added and solution of the albumin effected at room temperature or at 0°C . A stock solution, 33.3 per cent albumin, was thus prepared. Seventy-five cc. portions of these stock solutions were diluted with appropriate volumes of 1.2M sodium chloride and 1.2M fatty acid sodium salt to give final volumes of 100 cc. The experimental solutions thus obtained contained 25 per cent albumin⁶ and were 0.3M in total salt (exclusive of the small amount of added carbonate).

RESULTS

Before tabulating the cloud-point data, mention should be made of an important incidental observation. From cloud-point determinations on many different preparations, each one being studied at several different temperatures, it was found that a semilogarithmic plot of the data so obtained, logarithm C.P.⁶ against temperature, gave rise to a family of straight lines. This observation permits one in each and every case to calculate the 30-second cloud-point temperature and also the 65° cloud-point time. For comparative purposes, it is extremely helpful to express the results for different preparations or for different experimental conditions in a similar way, preferably as the 30-second cloud-point temperature. Here we might interpolate a second observation which is of some physico-chemical interest: The slope of the curve, log C.P. against temperature, is a function of protein concentra-

tion, the pH, and the nature of the added salt. Within limits, it is independent of the concentration of the added salt (sodium chloride).

The salts studied were sodium chloride, sodium acetate, sodium propionate, sodium butyrate, sodium valerate, sodium caproate, sodium caprylate, sodium phenylacetate, and sodium phenylbutyrate,—usually in an over-all salt concentration of 0.3M. The effects upon high temperature thermal stability (cloud-point) are presented in Table I. Many other substances

TABLE I
*Increase of thermal stability of serum albumin
by non-polar anions*

Salt added	Concentration M	pH*	30-second C.P. temperature
Chloride	0.3	6.78	67.29
Acetate	0.3	6.88	68.02
Propionate	0.3	6.88	71.48
Butyrate	0.3	6.97	75.09
Valerate	0.3	6.75	78.40
Caproate	0.3	6.78	79.96
Caprylate	0.3	A clear translucent gel formed between 80° and 100° ; no cloud formation.	
Phenylacetate	0.3		78.20
Chloride	0.15	7.26	63.1
Chloride Butyrate	0.15 } 0.15 }	7.28	71.7
Chloride Phenylacetate	0.15 } 0.15 }	6.84	76.9
Chloride Phenylbutyrate	0.15 } 0.15 }	7.15	81.5
Chloride Caprylate	0.15 } 0.15 }	6.68	81.8

* The values in this column are not for 25 per cent solutions of albumin but for solutions diluted 25-fold.

⁶ The effect of protein concentration on the cloud-point is inverse. Thus, a decrease in albumin concentration from 45 per cent to 5 per cent increased the 65° cloud-point about 35-fold.

⁶ Cloud-point is abbreviated as C.P.

have been studied, but the results are of little, if any, promise: Succinate, fumarate, lactate, glucose, alanyl glycine, glycerophosphate, and γ -globulin. It is of interest that the last named, in a concentration of 1.7 per cent, did not decrease the cloud-point of albumin in 0.3M chloride.

The effects of the non-polar anions on 50° and 57° thermal stability are presented in Figures 1 and 2, respectively.

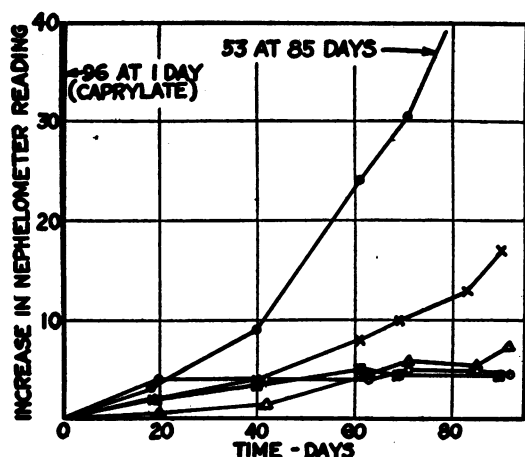


FIG. 1. EFFECT OF VARIOUS SALTS ON THE STABILITY OF PREPARATION 94-95 AT 50° C.

- 0.15M Chloride
- × 0.15M Chloride and 0.15M Chloride
- 0.15M Chloride and 0.15M Butyrate
- △ 0.15M Chloride and 0.15M Phenylacetate
- 0.15M Chloride and 0.15M Phenylbutyrate
- ⊠ 0.15M Chloride and 0.15M Caprylate

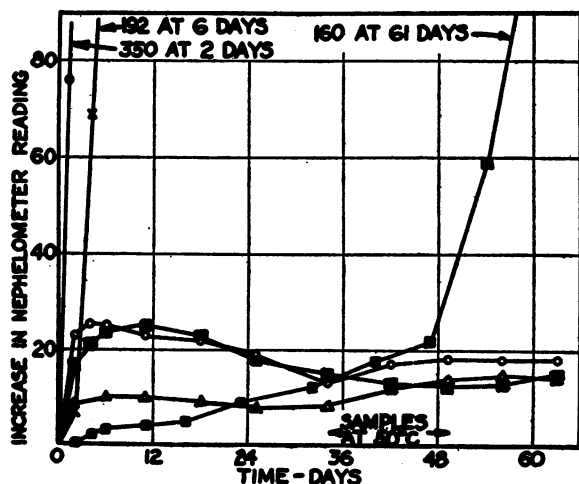


FIG. 2. EFFECT OF VARIOUS SALTS ON THE STABILITY OF PREPARATION 94-95 AT 57° C.

- 0.15M Chloride
- × 0.15M Chloride and 0.15M Chloride
- 0.15M Chloride and 0.15M Butyrate
- △ 0.15M Chloride and 0.15M Phenylacetate
- 0.15M Chloride and 0.15M Phenylbutyrate
- ⊠ 0.15M Chloride and 0.15M Caprylate

DISCUSSION

The marked stabilizing effect of the non-polar anions, studied on high temperature thermal stability (57° and cloud-point), are clearly evidenced by the results presented in Figure 2 and Table I, respectively. The experiments at 50° (Figure 1) are not yet terminated but are in qualitative agreement (except for caprylate) with the results observed at higher temperatures. With caprylate, the results are curious in that heat treatment (70°, 15 minutes) restores its stabilizing action as measured nephelometrically (at 50°). The increase in stability, conveyed by doubling the concentration of sodium chloride (Figures 1 and 2), confirms essentially the observations reported by Scatchard and co-workers, in the preceding paper of this series (2).

We are disposed to conclude that the added substances, in the comparatively high concentration used, do not inhibit denaturation of the protein molecule. The evidence now at hand, supported by some unpublished work, suggests that the albumin molecule begins to open out on heating and in doing so exposes more positively charged groups in side chains of amino acid residues. The stabilizing agent then becomes associated with the exposed amino groups ($R-NH_3^+$) through an electrostatic attraction with the carboxyl group of the added anion ($R'-COO^-$). The non-polar portion of the anion is attracted by the amino acid side chain in accordance with Van der Waals' forces; these, considered in the aggregate, would increase with increase in length of the carbon chain of the added substance. The protein-fatty acid anion complex must be regarded as possessing a greater solubility than denatured protein itself and of comparatively little tendency to flocculate out in coagula.

In so far as denaturation and coagulation are involved, we consider that interpretation of the phenomenon reported in this paper rests upon the theories of denaturation and coagulation now widely accepted among protein chemists and regarded as applicable to albumins particularly: an unfolding or opening out of the protein molecules in solution, followed, under certain conditions, by their flocculation, aggregation, or polymerization to give particles that are large

enough to scatter light or to form discrete coagula (7 to 18). This picture of denaturation as an opening out process is not applicable evidently to certain molecules such as myosin which in their native state are already quite extended (19).

SUMMARY

1. The thermal stability of serum albumin in 25 per cent solution has been studied at 50°, 57°, and cloud-point temperatures.

2. The capillary-tube cloud-point technic for studying protein coagulation is described.

3. Various non-polar anions are shown to enhance the thermal stability of human serum albumin, the effect increasing with increase in length of the fatty acid anion.

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