site on the protein. If acetic acid is bound to this site, perhaps through hydrogen bond formation, then subtle structural changes resulting in change in net charge may occur. However, if the amino acid is bound, then the positively charged amino group might interact electrostatically with a neighboring negatively charged group on the protein, thereby preventing the structural changes from occurring. In this event, steric factors would be expected to influence the strength of the electrostatic interaction and thus the action of the amino acid. The smaller effect of glycine on the electrophoretic composition of γ-globulin at *p*H 4 as compared to ovalbumin at the same *p*H can be understood in terms of difference in electrostatic interaction between the net charge on the protein and the amino acid. Since at this *p*H γ-globulin carries a much greater net positive charge than ovalbumin, the electrostatic repulsion between the positively charged protein and the acid form of the amino acid would also be greater and the extent of glycine binding would be less.

It is interesting to compare the effect of acetic acid and amino acids on the electrophoretic behavior and on the kinetics of heat denaturation of proteins. Gibbs[5] has found that the rate of denaturation of human plasma albumin at 55–65° is greater in the presence than in the absence of acetate buffer. Also, the heat of activation is considerably lower when acetate is present. In contrast, glycine[6] has no influence on the denaturation of albumin in the presence of chloride but absence of acetate. It would appear that at least some of the isomeric states of serum albumin in acetate buffer are more heat labile than the state of the protein in the absence of acetate. Ballou, Boyer, Luck and Lum[7] have found that fatty acid anions increase the heat stability of relatively concentrated solutions of albumin at nearly neutral *p*H in the restricted sense of inhibiting aggregation of presumably denatured protein. The effectiveness of the acid anions increases with increase in chain length.

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[CONTRIBUTION FROM THE ROBERT W. LOVETT MEMORIAL LABORATORIES FOR THE STUDY OF CRIPPLING DISEASES, MASSACHUSETTS GENERAL HOSPITAL, AND THE DEPARTMENT OF MEDICINE, HARVARD MEDICAL SCHOOL]

Characterization of an "Electrophoretically Homogeneous" Human Serum Albumin[1]

BY K. SCHMID

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A hitherto unknown form of albumin has been isolated from Cohn's Fraction VI of pooled normal human plasma. It was crystallized and partially characterized. Its most outstanding property is the homogeneity by electrophoresis at *p*H 4.0 which is lost after treatment with cysteine and deionizing with ion-exchange resins.

In the course of a systematic study of the very soluble proteins of normal human plasma,[2,3] a new form of albumin was discovered. It is distinguished by special solubility properties and, particularly, by its electrophoretic homogeneity in *p*H 4.0 acetate buffer.

Experimental

The starting material for these investigations was the supernatant solution of Fraction V derived from pooled normal human plasma which had been fractionated according to Method 6.[4] Throughout the fractionation work, the temperature of the solutions was kept at −5°. The protein of the starting material was concentrated with zinc hydroxide, and the resulting protein–zinc hydroxide paste was decomposed at *p*H 5.8.[2] Insoluble material, the protein of which consisted essentially of albumin identical to that of Fraction V,[4] was removed. Precooled 1M $BaAc_2$ was added to the supernatant solution to give a concentration of 0.02 *M*. The formed barium precipitate was centrifuged, dissolved by addition of a precooled, aqueous solution of neutralized ethylenediaminetetraacetic acid, and subsequently dialyzed and lyophilized.

The resulting protein fraction designated as Fraction VI-2,[2] was comprised of approximately 90% albumin as judged by electrophoresis in *p*H 8.6 diethyl barbiturate buffer. The corresponding subfraction obtained from any other runs so far investigated was essentially free of albumin. The major component of our fraction crystallized easily at −2° as a Pb^{++}-complex[5] from a 7% protein solution which, in addition, contained 10% methanol and 10% acetone. The *p*H of the solution was not controlled. The crystals were washed with ice-water, dissolved by adding sodium citrate, dialyzed and lyophilized. The recrystallized protein appeared homogeneous on electrophoretic and ultracentrifugal analyses: the electrophoretic mobility in *p*H 8.6, Γ/2 0.1 diethyl barbiturate buffer was 6.2×10^{-5} cm.²/volt. sec. and the sedimentation constant of a 1.4% solution in 0.15 *M* NaCl was 4.6 *S*. The protein was devoid of a component with a sedimentation constant of 6 *S* corresponding to that of the albumin dimer and did not seem to contain free sulfhydryl groups judging from the negative nitroprusside reaction and its inability to form the mercurial dimer.[6,7] Immunochemical determination using an antiserum against human Fraction V-albumin indicated essentially pure serum albumin. The carbohydrate content of the crystallized protein proved negligible. In contrast to the albumin of Fraction V or human mercaptalbumin, the above-described albumin remained homogeneous

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on electrophoresis at *p*H 4.0 in Γ/2 0.1 acetate buffer[8] (Fig. 1a). Its electrophoretic mobility amounted to 2.4 × 10^{-5} cm.²/volt-sec.[9] Under identical conditions, ordinary human serum albumin (Fraction V) split in two components[10-12] (Fig. 1c). The crystallized albumin appeared also homogeneous at *p*H 4.2 (acetate, Γ/2 0.1) and at *p*H 2.8 (NaCl-HCl, Γ/2 0.1). At *p*H 3.6 (acetate, Γ/2 0.1), a minor, slower moving component was observed. The dialysis times prior to electrophoresis were 2 and 30 hours, respectively.[13] The solubility of this protein was measured

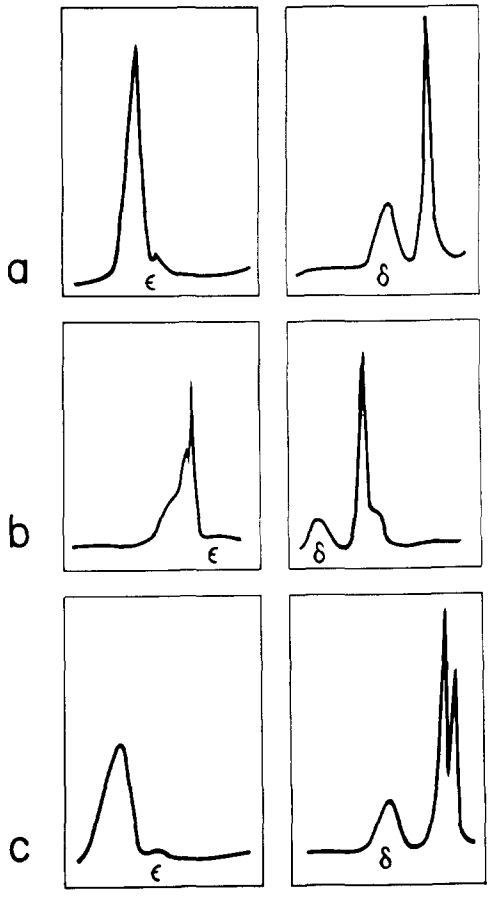

Fig. 1.—Schematic line drawings of the electrophoretic patterns of the crystallized human serum albumin in *p*H 4.0 acetate buffer of Γ/2 0.1. Time of the runs 60 minutes using a Perkin-Elmer electrophoresis apparatus. Pictures a before and pictures b after treatment with cysteine and ion-exchange resins. For comparison, pictures c, normal human serum albumin of Fraction V. Anomalies on the ascending patterns designated as δ and those on the descending as ϵ.

over a *p*H range from 5.0 to 6.0 in the presence of zinc and barium ions and compared with that of human mercaptalbumin (Fig. 2). The extinction coefficient of a neutral solution of the crystallized albumin measured at 278 mμ equaled to 6.2. However, the ultraviolet absorption curve did not appear typical of a simple protein (Fig. 3, curve 1) because the minimal absorption was just below the value of the maximal and, furthermore, was observed at 272 mμ instead of 253 mμ.

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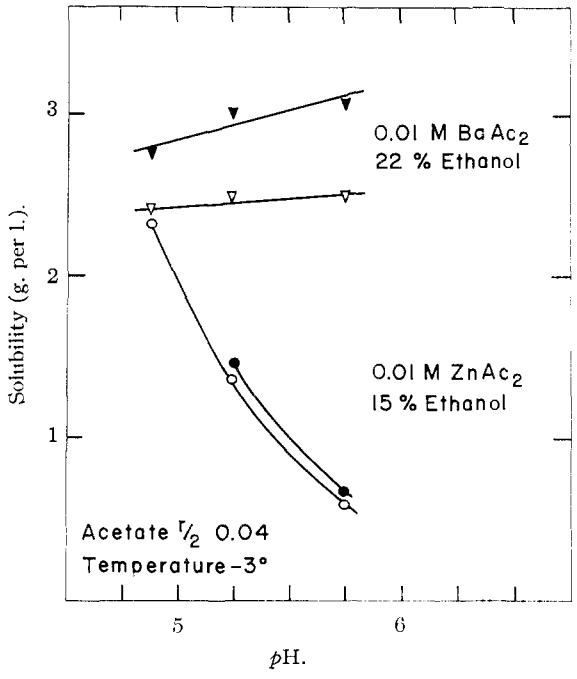

Fig. 2.—Solubility of the crystallized, "electrophoretically homogeneous" human serum albumin compared with that of human mercaptalbumin. The full signs represent the solubility values of the crystallized albumin and the open—those of mercaptalbumin.

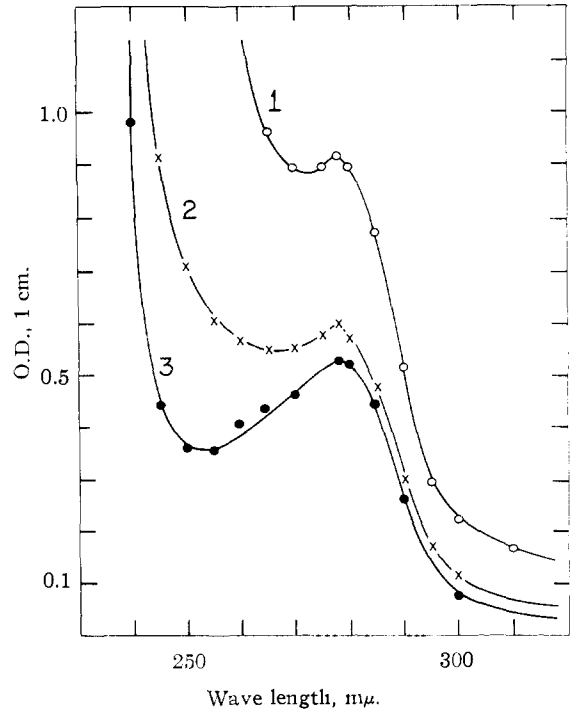

Fig. 3.—Ultraviolet absorption of the crystallized, "electrophoretically homogeneous" human serum albumin, curve 1. Curve 2 represents the ultraviolet absorption after incubation with cysteine and curve 3 that after deionizing with ion exchange resins. The protein concentration of the solutions was 0.15, 0.10 and 0.10%, respectively.

A neutral solution of the crystallized protein (80 mg. dissolved in 5.0 ml. of dist. water) was mixed with cysteine hydrochloride (3.0 mg. in 0.2 ml. of water) to lower its *p*H to 5.0. As soon as this *p*H value had been obtained, the color of the solution changed from almost colorless to brown, accompanied by evolution of hydrogen sulfide. After standing at room temperature the colored compound separated. No insoluble material was formed, if *p*H 4.0 acetate buffer was used to change the *p*H of the protein solution. The ultraviolet absorption curve of the protein (Fig. 3, curve 2), all of which remained in the colorless supernatant solution indicated that only part of the impurities had been removed. Subsequently, the protein solution was deionized at +2° by passing through a resin column as described by Oncley and Dintzis.[14,15] During this operation the remainder of the impurities was removed as judged by the corresponding ultraviolet absorption curve (Fig. 3, curve 3). This curve showing a maximum at 278 mμ and, particularly, a minimum at 253 mμ, was very similar to that of normal human albumin. The extinction coefficient of the protein was now 5.5, identical to that of Fraction V-albumin. Examination of the ultraviolet absorption curves obtained at *p*H 13[18] indicated that the tyrosine and tryptophan content of the albumin described here and that of Fraction V were essentially the same. Electrophoretic investigation at *p*H 4.0 in Γ/2 0.1 acetate buffer of the protein obtained after passage through the column revealed three components (Fig. 1b); however, at *p*H 8.6 in Γ/2 0.1 diethyl barbiturate buffer it remained homogeneous. Judging from the ultracentrifugal homogeneity (1% dimer), the protein had not been denatured during the incubation with cysteine and the deionization with ion-exchange resins. In preliminary experiments it was found that this protein was at least partially capable of forming a dimer (40%) with $HgCl_2$.[6,7]

Discussion

The albumin described in this paper is distinguished by three outstanding properties as compared with those of the albumin of Fraction V: solubility differences, electrophoretic homogeneity in *p*H 4.0 acetate buffer and the conversion to the heterogeneous form. Of even greater importance are the theoretical implications.

The solubility characteristics were as follows. The "electrophoretic homogeneous" albumin was soluble at −3° in a solution whose *p*H was 5.8 and which contained 19% ethanol and was saturated with $ZnAc_2$. It was precipitated from this solution by addition of $BaAc_2$. On the basis of these properties the separation of the two forms of albumin from each other could be accomplished. At low ionic strength, −3° and *p*H 5.8 (Fig. 2) the solubility of the Zn^{++}-complex of the albumin described here was approximately the same as that of mercaptalbumin. However, the Ba^{++}-complexes of these two proteins differed significantly in their solubility, particularly at relatively neutral *p*H values. The dependence of the solubility upon *p*H of the Ba^{++}-salts of these two plasma constituents contrasted that of their Zn^{++}-complexes.

The results of the present investigations further demonstrate that, in contrast to human serum albumin of Fraction V, the albumin described in this paper remained homogeneous on electrophoretic analysis at *p*H 4.0.[17] However, following removal of a specific part, this form of serum albumin revealed a heterogeneity somewhat similar to that of the Fraction V-albumin.

The electrophoretic heterogeneity of serum albumin in acid solutions has been attributed to a reversible expansion of the protein molecule due to unfolding of the polypeptide chain[18] as a result of rupture of "salt-bridges" between side chain groups of the protein molecules.[19] Recently, the heterogeneity has also been explained on the basis of isomerization[9,20] and interaction with acetate ions.[13] Acetate binding may modify this phenomenon,[13] but as pointed out by the above-mentioned authors the principal factor governing the reaction leading to heterogeneity seems to be the *p*H value of the albumin solution.

The crystallized, "electrophoretically homogeneous" albumin would seem to contain a "blocking agent" which is removable with cysteine and is, therefore, thought to be bound to specific sites of the protein. As long as the "blocking agent" remains uncharacterized[21] and until it is known whether or not this albumin undergoes structural expansion in acid solutions, there is room for speculation.[22] The existing theories require that the unfolding of the polypeptide chain or the isomerization of Fraction V-albumin must be accompanied by an increase in the positive net charges with subsequent appearance of the electrophoretic heterogeneity. The present work indicates that our albumin preparation, when analyzed in acid solutions, does not exhibit heterogeneity. Three explanations for this observation may be considered: first, it is conceivable that the relatively weak and *p*H-dependent salt-bridges of Fraction V-albumin were modified by the "blocking agent" to more stable bonds.[21] The spacial arrangement of these bonds must be such that unfolding of the polypeptide chain cannot take place. Secondly, interactions other than reversible formation and rupture of salt-bridges may be involved in a complex reaction system leading to electrophoretic heterogeneity. One of these reactions might have been modified by the "blocking agent," thereby preventing the appearance of the electrophoretic heterogeneity. Thirdly, new bonds[18,19,22] were formed by which the "blocking agent" acted as stabilizer with respect to the electrophoretic behavior of serum albumin at acid *p*H values.

The relationship between the "electrophoretically homogeneous" albumin and the albumin of Fraction V may be suggested by the results of the following investigations: analysis of the albumin fractions obtained by chromatographic separation of human serum or Fraction V on modified cellulose revealed three albumins[23] which differed in their electrophoretic properties at *p*H 4.0.[24] Recently,

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(24) One of these fractions revealed 3 components upon electrophoretical analysis at *p*H 4.0. Private communication from Dr. H. A. Sober.

Tiselius[25] reported on the separation of human serum albumin in three components using calcium phosphate. A possibly related observation has been made by Lapresle[26] who showed that, following enzymatic digestion, human serum albumin consisted of three immunochemically similar proteins. Furthermore, Luetscher[10] noted that the albumin of normal human plasma splits into two components upon electrophoretic analysis at *p*H 4.0. The same protein fraction derived from pathological plasma and investigated under the same conditions revealed also two components but their ratio varied depending on the type of disease. Thus, it would appear that the crystallized, "electrophoretically homogeneous" albumin may be identical with one of the three individual components of normal human serum albumin.

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BOSTON, MASS.

[CONTRIBUTION NO. 2199 FROM THE GATES AND CRELLIN LABORATORIES OF CHEMISTRY, CALIFORNIA INSTITUTE OF TECHNOLOGY]

A Quantitative Study of the Hydrolysis of Human Dinitrophenyl(DNP)globin: The Number and Kind of Polypeptide Chains in Normal Adult Human Hemoglobin

BY HERBERT S. RHINESMITH,[1] W. A. SCHROEDER AND LINUS PAULING

RECEIVED APRIL 11, 1957

A quantitative investigation of the partial hydrolysis of DNP-globin in refluxing 6 *N* hydrochloric acid has led to an explanation of our earlier conclusion that normal adult human hemoglobin contains a non-integral number, 3.6, of N-terminal valyl residues per molecule. It is now concluded that 4 N-terminal residues are present. Moreover, it has been found that the molecule contains two kinds of polypeptide chains, with respect to the N-termini. Under the above hydrolytic conditions the N-terminal valyl residues are released as DNP-val-leu almost quantitatively from two chains (A chains) within 15 min. On continued hydrolysis the other two chains (B chains) release DNP-valine. No N-terminal peptides originating from the B chains have been definitely identified.

Introduction

In a previous paper[2] we reported a non-integral value for the number of N-terminal valyl residues in normal adult human hemoglobin, and suggested that this finding raised questions about the molecular weight and homogeneity of hemoglobin. During that investigation DNP-val-leu was found to be the only peptide that could be isolated in significant amount from the partial hydrolyzates of DNP-globin. The isolation of DNP-val-leu accords qualitatively with the findings of Brown,[3] who reported a maximum quantity of DNP-val-leu corresponding to 2.5 chains per molecule of DNP-hemoglobin.

The original objective of the present investigation was the isolation and identification of N-terminal peptides other than DNP-val-leu. Although what may be other N-terminal peptides have been isolated, the most striking feature of the first experiments was the almost instantaneous release of much DNP-val-leu and the slow release of DNP-valine during hydrolysis in refluxing 6 *N* hydrochloric acid. It was evident that the maximum amount of DNP-val-leu is by no means equivalent to all of the DNP-valine that is ultimately released by continued hydrolysis. This fact suggested that at least two types of N-terminal sequences are present in human hemoglobin. The results of the quantitative study of the hydrolysis presented in this paper support this initial conclusion.

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Experimental

Preparation of DNP-globin.—Carbonmonoxyhemoglobin, twice crystallized by the method of Drabkin,[4] was dinitrophenylated at 40° and *p*H 9.0 by the method of Levy and Li.[5] The heme was removed by the method of Anson and Mirsky[6] and the purified DNP-globin was equilibrated in the air to constant weight. Experimental details for preparing the DNP-globin used in this work were described.[2]

Partial Hydrolysis of DNP-globin.—Samples varying in weight from 50 to 500 mg. were employed for the hydrolyses. A 10-ml. portion of 6 *N* hydrochloric acid was used for each 100 mg. of sample. Foaming that occurred during the hydrolysis could be decreased materially if the DNP-globin was wetted thoroughly with acid by allowing it to stand at room temperature for one hr. with occasional swirling. The hydrolysis was carried out by refluxing on a hot plate except for the 15-min. runs, for which a pre-heated oil-bath was used. The time necessary to bring the solution to boiling was not counted as part of the time of hydrolysis. At the end of the period of hydrolysis the reaction was stopped by immersion in cold water and the hydrolyzate was extracted with 4 × 25 ml. of ether. The combined ether extracts were washed with 4 × 5 ml. of distilled water, each washing containing one drop of 6 *N* hydrochloric acid. The ether was evaporated, the residue was taken up in acetone, the acetone was evaporated, and the residue was reserved for chromatography.

Samples of DNP-globin were hydrolyzed for 0.25, 0.5, 1, 2, 4, 7, 12.5 and 22 hr., respectively; the 2-hr. and 4-hr. runs were in duplicate, and the 0.25-hr. run in triplicate. The result for 22 hr. of hydrolysis is the average of the eleven runs reported previously.[2]

In these hydrolyses the DNP-globin was not completely dissolved until about 4 hr. had elapsed. The insoluble portion in the 15-min. hydrolyzates was appreciable and could not be neglected in the quantitative evaluation of the data. The experiments described in the following section were accordingly made.

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