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The Aggregation of Bovine Plasma Albumin at Low *p*H[1]

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The aggregation of bovine plasma albumin below its isoionic *p*H has been subjected to further study. Results of the investigation substantiate previous conclusions that aggregation is not significant in 0.02 *M* NaCl. However, in 0.1 *M* NaCl aggregation, predominantly as dimerization, is maximal near *p*H 3.3 and diminishes rapidly as the *p*H is lowered below 3 or raised above 3.5. Both the rate and extent of dimerization appear to be smaller for albumin which had not been treated to remove the lipid impurity (which is present in the albumin) than for albumin which had been "defatted" prior to analysis. The reconversion of dimer to monomer in solutions which had stood for two or more days was accomplished only by prolonged dialysis against a low *p*H (~2) solution or against 0.02 *M* cysteine (*p*H 3.3–3.4). It is proposed (1) that native albumin must first be transformed into a more "aggregatable" isomeric or slightly expanded form before aggregation becomes feasible, and (2) the aggregation (dimerization) process is accomplished in two stages, the first of which is readily *p*H-reversible, but that the subsequent formation of intermolecular disulfide bonds stabilizes the dimer towards *p*H changes.

Introduction

The recent interest in the structural modifications of plasma albumin at low *p*H has produced numerous scattered observations of molecular aggregation and some controversy has resulted. The status of the available data was reviewed by one of us[3] with the conclusion that aggregation does not take place at low ionic strength. There is considerable evidence that lipid material is liberated from albumin in low *p*H solutions[4–10] and since the liberation of this material produces noticeable turbidity, it seems probable that this turbidity has sometimes been mistaken for protein aggregation. On the other hand there have been repeated observations of what is clearly aggregation of plasma albumin under conditions of higher ionic strength (0.1 and above.)[11] Bro, *et al.*,[12] have examined in detail the aggregation of bovine plasma albumin (BPA) in the presence of chloride ion at an ionic strength of 0.1. They postulated that BPA dimerizes by means of an inter-molecular thiol–disulfide exchange reaction and their results indicate clearly that the resulting dimer differs from both the mercury dimer of mercaptalbumin[13] and the disulfide dimer which is obtained by iodine oxidation of this mercury dimer.[14] However, the results of their investigation show considerable variability, the basis of which was not determined.

The initial purposes of the present investigation were several fold, including: (1) Verification that the material which is released at low *p*H is primarily fatty acid, (2) substantiation that aggregation (primarily as dimerization) takes place at low *p*H in 0.1 but not in 0.02 *M* Cl^- and (3) elucidation of the cause of the lack of reproducibility in the experiments of Bro, *et al.*[12]

(1) This investigation was supported in part by the National Cancer Institute, National Institutes of Health, Grant C-2248, and by the National Science Foundation, Grant G-1953.

(2) Fellow of the Visking Corporation, 1957–1958.

(3) J. F. Foster, *J. Phys. Chem.*, **61**, 704 (1957).

(4) J. T. Yang and J. F. Foster, THIS JOURNAL, **76**, 1588 (1954).

(5) C. Tanford, J. G. Buzzell, D. G. Rands and S. A. Swanson, *ibid.*, **77**, 6421 (1955).

(6) M. D. Sterman and J. F. Foster, *ibid.*, **78**, 3562 (1956).

(7) P. Bro and J. M. Sturtevant, *ibid.*, **80**, 1789 (1958).

(8) H. M. Dintzis, Ph.D. Thesis, Harvard University, 1952.

(9) M. Champagne, *J. chim. phys.*, **57**, 393 (1957).

(10) E. J. Williams and J. F. Foster, THIS JOURNAL, **81**, 865 (1959).

(11) For a review of the pertinent papers see ref. 12.

(12) P. Bro, S. J. Singer and J. M. Sturtevant, *ibid.*, **80**, 389 (1958).

(13) W. L. Hughes, Jr., *ibid.*, **69**, 1836 (1947).

(14) R. Straessle, *ibid.*, **76**, 3138 (1954).

Experimental

Crystalline Armour brand BPA was used to prepare stock protein solutions. Sample solutions for ultracentrifugal analysis were prepared from these deionized and defatted stock solutions, 1 *N* NaCl and 0.1 *N* HCl, and deionized water. The sodium salt of recrystallized ethylenediaminetetracetic acid (EDTA), crystalline ascorbic acid, crystalline Sigma brand cysteine hydrochloride and crystalline stearic acid were employed at different stages of the investigation as possible aggregation inhibitors. All chemicals, unless noted otherwise, were reagent grade and were used without further purification.

All ultracentrifugal experiments were carried out in Kel F cells using a Spinco Model E analytical ultracentrifuge equipped with thermistor temperature control and phase-plate optical system. Light-scattering experiments were performed with a calibrated Brice–Phoenix Universal light-scattering photometer. All *p*H measurements were made with a Beckmann Model G *p*H-meter and protein concentrations were determined using a Beckmann Model DU spectrophotometer; the extinction coefficient of BPA ($E^{1\%}_{1 cm}$) at 279 mμ was taken as 6.67.[15] All ultracentrifuge runs were made at 25° and 59,780 r.p.m. while light-scattering and *p*H measurements were made at room temperature. Solutions were clarified for light-scattering by filtration, while cold, through an ultra-fine sintered glass filter or by centrifugation in a Servall centrifuge at 5°. Infrared analyses of the lipid material and of crystalline stearic acid were performed with a Perkin–Elmer Model 21 spectrophotometer using the KBr pellet technique.[16] Dialysis was accomplished at 5° utilizing mechanical agitation. In each dialysis there were three changes of the dialysate. The volume ratio of the solution being dialyzed to the dialysate was approximately 1:20.

The method for removal of the lipid material has been described in a previous publication.[10] Essentially the method consists in lowering the *p*H of a stock BPA solution (1–2%) to *p*H 3 and allowing the solution to stand at 5° for two to three days. The insoluble material is then filtered off and the protein filtrate deionized by passing it through a mixed bed ion exchange column.[8] It is noteworthy that this procedure for removal of the lipid material is not always 100% efficient. To isolate the lipid impurity for analysis, the material on the sintered glass filter was first washed with several portions of dilute HCl, then with deionized water, to remove any adhering protein. Finally, the residue was eluted with acetone and thoroughly dried *in vacuo* at 40°.

The percentage of dimer in the low *p*H solution was estimated by dividing the area of the faster-sedimenting component by the total area. The total area represented the sum of the areas of the peaks corresponding to monomer and dimer plus a small amount of a fast-sedimenting third component (when present). This third peak was observed only in cases where there was a significant amount of the dimer. To obtain these respective areas, magnified projections of the ultracentrifuge patterns were traced and planimetered. In cases where the dimer peak was not sufficiently separated from the monomer peak, a graphical method of symmetrical

(15) J. F. Foster and M. D. Sterman, *ibid.*, **78**, 3656 (1956).

(16) R. D. Meiklejohn, R. J. Meyer, S. M. Aronovic, H. A. Schuette and V. M. Meloch, *Anal. Chem.*, **29**, 329 (1957).

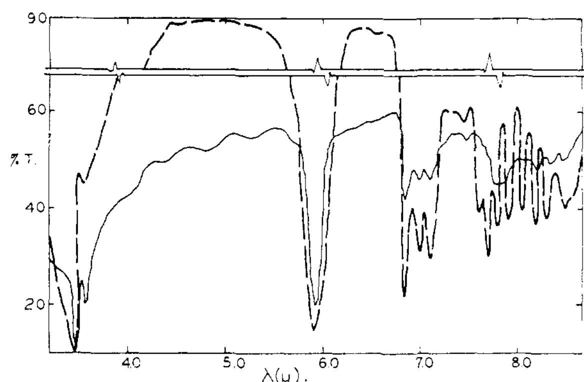


Fig. 1.—Infrared spectra in KBr pellets: - - -, stearic acid and ——, material isolated from BPA by low pH treatment.

resolution[17] was employed. All areas were corrected for radial dilution but not for the Johnston–Ogston effect which was assumed to be of the order of the relative experimental error associated with calculation of the percentage of dimer ($\pm$ 5%).

Results and Discussion

Various investigators have observed that low pH BPA solutions become turbid upon standing even at low temperature. The ensuing turbidity is believed by some to be due to protein aggregation with subsequent precipitation[18] while others are of the opinion that the material responsible for the turbidity is a fatty acid[4–10] or ketone[7] impurity. In an attempt to clarify this question, the material responsible for the turbidity of low pH solutions was isolated and analyzed utilizing infrared spectrophotometry[16] and methanolic alkaline titration.[19]

It has been observed that this material sediments to the top and not to the bottom upon centrifugation[4] which appears in agreement with the contention that it is of the nature of a lipid or lipoprotein complex.[9] The infrared pattern of the deproteinized material is very similar to the pattern of stearic acid (Fig. 1). The band structure near 7.0 μ is good evidence that the material is of the nature of an acid rather than a ketone. Meiklejohn, *et al.*,[16] have proposed that the number of bands due to CH_2 wagging (7.4–8.4 μ) is equal to one-half the number of carbon atoms in the fatty acid (excluding the carboxyl carbon). The isolated impurity shows a multiplicity of bands in this region comparable to the eight shown by stearic acid. The relatively poor resolution of these bands suggests that this material consists of an homologous mixture of acids. The value of the neutralization equivalent, 298, is in good accord with that of stearic acid and there was no detectable uptake of iodine by the material. It is estimated that the amount of the impurity corresponds to not over one to two moles per mole of protein.

It is important to note that no evidence for aggregation was observed in the ultracentrifugal patterns of BPA in 0.02 M NaCl (see, for example,

(17) J. P. Johnston and A. G. Ogston, *Trans. Faraday Soc.*, **42**, 789 (1946).

(18) M. E. Reichman and P. A. Charlwood, *Can. J. Chem.*, **32**, 1092 (1954).

(19) E. J. Cohn, W. L. Hughes, Jr., and J. H. Weare, THIS JOURNAL, **69**, 1753 (1947).

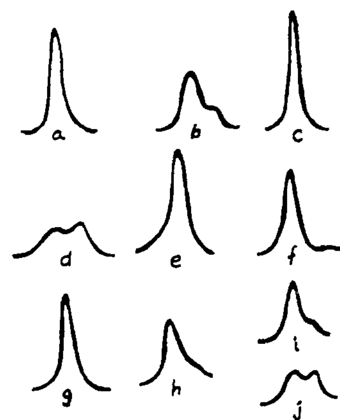


Fig. 2.—Representative tracings of ultracentrifuge sedimentation patterns. The protein concentration was 1.0% in all cases. All runs on defatted protein and in presence of 0.10 M chloride unless otherwise noted: a, pH 3.23, 0.02 M chloride, aged 2–3 days, BPA not defatted; b, pH 3.22 after aging several days; c, sample permitted to aggregate at pH 3.36 followed by dialysis for 12 days against 0.02 N HCl; d, sample aggregated at pH 3.36 followed by dialysis against 0.1 M NaCl for over one week; e, Sample aggregated at pH 3.36 followed by dialysis against 0.02 M cysteine hydrochloride for 12 days; f, pH 3.10, sample aged two days in presence of saturated EDTA; g, pH 3.32, BPA not defatted, aged three hours; h, pH 3.34, solution g three days later; i, pH 3.32, aged three hours; j, pH 3.33, solution i three days later.

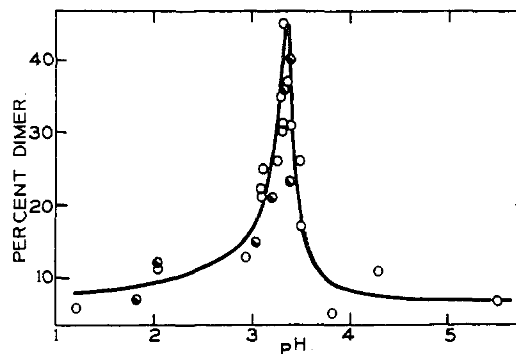


Fig. 3.—Dependence of percentage of BPA dimer on pH: O, 1% and ●, 0.5% solutions BPA.

Fig. 2a), even in the pH range where aggregation was optimal at higher ionic strength (Fig. 3). This is in complete accord with previous observations.[3]

Figure 3 summarizes the pH-aggregation relationship in 0.10 M chloride and shows aggregation to go through a distinct maximum near pH 3.3. These results are in good accord with those previously published by Bro, Singer and Sturtevant[12] except that the present results appear to be much more consistent and reproducible than the results which those authors reported. The observation that aggregation is insignificant at very low pH is in agreement with the light-scattering results of Reichman and Charlwood[18] who found that at pH 1.9, 0.1 and 0.25% BPA solutions exhibited no evidence of aggregation in 0.1 M Cl^-. To verify these results at higher BPA concentrations (0.5

and 1.0%) the turbidity and dissymmetry of a pH 1.72, 0.1 M Cl^- solution containing BPA (pretreated to remove the fatty acid) was determined 10 hr. after preparation of the solution. These measurements were then repeated 24 and 48 hr. later. No change in dissymmetry with time was noted while the turbidity exhibited a very slight decrease. A similar experiment was performed with 0.02 M Cl^- solutions. Again, no significant time dependence of dissymmetry or turbidity was evident.

Gorbacheva, *et al.*,[20] have reported that EDTA in a concentration of approximately 5×10^{-4} M and in the presence of 2×10^{-3} ascorbic acid (AA) prevented aggregation of BPA in 6 M urea at pH 7.6 (1% BPA, 0.1 M borate). In view of these results it seemed advisable to consider the possibility that the variability inherent in the experiments of Bro, *et al.*,[12] was a consequence of metal ion contamination. Bro and co-workers had recognized the possible role of metal ions in the aggregation process and in fact determined, by emission spectrographic analysis, that crystalline BPA (Pentex) contains small amounts of metal impurities. Most of these impurities should be removed from the protein in the deionization step and estimates made from analytical data on the deionized water and the reagents used indicated that metal contamination should not exceed about 10^{-6} M. Bro, *et al.*, did not deionize either the protein or the water they employed. They considered the amount of contamination by metals to be too low to account for the dimerization observed. It is possible, however, that even trace contamination might be important catalytically. To test the hypothesis that dimerization is accomplished by a thiol–disulfide reaction which is catalyzed by trace amounts of metals (copper, iron, etc.),[21] experiments were performed with EDTA, the results of which are summarized in Table I. Clearly this metal chelating agent does repress aggregation; however, the EDTA concentration which is required seems unusually high in view of the very low level of metal ion contamination. One might tentatively conclude that the repression of aggregation by EDTA is due to some factor other than metal chelation and that trace contamination by metal ions is not an important factor in the present studies.

It is evident from Table I that the addition of ascorbic acid or stearic acid does not inhibit aggregation. In fact a high concentration of ascorbic acid tends to promote aggregation.

An interesting aspect of this investigation was the observation that the rate of aggregation is markedly dependent on whether the protein has been pretreated to remove the fatty acid impurity. This is brought out by examination of Table II. The relatively consistent results shown in Fig. 3 were all obtained with pretreated protein while Bro, Singer and Sturtevant did not employ such pretreatment.[12] The effect of pretreatment could be due to the unmasking of the aggregation sites as a consequence of the removal of the fatty acid or alternatively to an adventitious structural alteration resulting from the low pH treatment. In view of results of Bro and Sturtevant[7] which show the release of fatty acid from the protein to be a relatively fast process (a few hours) compared to the time required to reach optimal aggregation (1 to 2 days) the latter alternative seems more likely. The fact that addition of colloidal stearic acid caused no repression of aggregation also suggests that aggregation results from structural modification of the protein molecule as a consequence of the loss of fatty acid and not from liberation of the fatty acid *per se*. However, it is also possible that the added stearic acid does not localize in the same sites which were occupied in the native protein. There is evidence that removal of fatty acid by alternative means may also promote aggregation of BPA. Other investigators have concluded that there are two sites in the BPA molecule which may bind fatty acid tightly[22,23] and that the tightly bound fatty acid is not completely removed when the protein is deionized[24] but is removed only when the protein is extracted with certain organic solvents (ethanol,[14] methanol,[19] isooctane[24]). It is interesting, in regard to the use of these solvents, that sedimentation patterns of BPA which had been treated with methanol to remove the fatty

TABLE I

EFFECT OF EDTA, ASCORBIC ACID (AA) AND STEARIC ACID (SA) ON THE AGGREGATION OF BPA IN PRESENCE OF 0.1 M CHLORIDE

pH	Aging time	% Dimer[a]	Remarks
3.06	2 days	23	No inhibitor present
3.07	2 days	23	No inhibitor present
3.04	2 days	14	2.1×10^{-3} M EDTA present
3.04	2 days	8	2.6×10^{-3} M EDTA present[b]
3.04	2 days	~40	4.0×10^{-2} M AA present
3.12	1–2 days	25	No inhibitor present
3.10	2 days	5	2.6×10^{-3} M EDTA present[b]
3.12	2 days	23	4.0×10^{-3} M AA present
3.32	3 hr.	31	No inhibitor present
3.32	3 hr.	25	SA present[c]
3.32	3 hr.	29	SA present[d]

[a] In 1% BPA solution. [b] Solution saturated with EDTA. [c] Crystalline stearic acid added at pH 3.32. [d] Crystalline stearic acid added to a BPA solution at pH 5.8 and a pH 3.32 solution prepared from this solution after it had been stirred at room temperature for several days.

TABLE II

EFFECT ON THE RATE OF AGGREGATION OF PRETREATING BPA TO REMOVE THE FATTY ACID IMPURITY

pH	Aging time	% Dimer[a]	Remarks[b]
3.32	2 hr.	28	BPA pretreated
3.32	3 hr.	8	No pretreatment
3.32	2 days	35	BPA pretreated
3.34	3 days	25	No pretreatment
3.32	7 days	31	BPA pretreated
3.39	9 days	24	No pretreatment

[a] For 1% BPA solutions. [b] Measurements were made on two solutions, one containing pretreated BPA, the other containing native BPA.

(20) L. Gorbacheva, S. E. Bresler and Y. Frenkel, *J. Biochem. U.S.S.R.*, **22**, 66 (1957).

(21) J. C. Bailar, "The Chemistry of Coordination Compounds," Reinhold Publishing Corp., New York, N. Y., 1956.

(22) D. S. Goodman, THIS JOURNAL, **80**, 3892 (1958).

(23) D. S. Fredrickson and R. S. Gordon, Jr., *Physiol. Rev.*, **38**, 585 (1958).

(24) B. S. Goodman, *Science*, **135**, 1296 (1957).

acid revealed the presence of approximately 30% of a faster-sedimenting component (which presumably corresponds to BPA dimer).[19]

Monomer and dimer sedimentation coefficients ($S_{20,w}$) were calculated for 1% BPA solutions using BPA which had been pretreated. The monomer values were in good agreement with those of Bro, *et al.*,[25] but the dimer values were somewhat low, possibly due to the lack of precision in locating the center of the dimer peaks in the ultracentrifugation patterns. Because of the good agreement in the case of the monomer between values for pretreated BPA and those of Bro, *et al*, who did not pretreat their BPA, it appears that the pretreatment of BPA has not altered the gross structure of the protein to any great extent.

Some experiments were performed to ascertain the extent to which the aggregation process is reversible. A solution whose *p*H had been adjusted to 1.23 and allowed to stand for 2–3 days showed no significant decrease in the amount of dimer while there was evidence that the amount of dimer had been reduced only slightly in a solution which had been adjusted to *p*H 10.3. Also when solutions containing dimer were dialyzed for 8–12 days against 0.1 or 0.02 *M* Cl^- solution at *p*H values ranging from 3.4 to 7, no decrease in the percentage of dimer was noticeable (Fig. 2d). These results indicate that the aggregation is essentially irreversible over a rather broad range of *p*H. On the other hand, dialysis for 12 days against 0.02 *N* HCl did result in obliteration of all but a trace of the dimer (Fig. 2c). Dialysis against 0,02 *M* cysteine hydrochloride for 12 days at *p*H 3.36 eliminated essentially all of the aggregate (Fig. 2e) although dialysis for two days against 0.01 *M* cysteine hydrochloride at *p*H 2.15 resulted in no decrease in the amount of dimer.

Bro and co-workers[12] have postulated that the aggregation process involves an intermolecular thiol–disulfide reaction. They have observed, however, that dimerization is reversed (in some instances) when the *p*H is raised above 3.5. They also observed reversal when mercuric chloride was added to a solution containing dimer. Moreover, cysteine or thioglycolate did not dissociate the dimer although passage through a thioglycolate column did do so. The seemingly ambiguous nature of these results coupled with those of the present investigation (see preceding paragraph) indicate that although the aggregation may involve the formation of intermolecular –S–S–bonds, this is but one phase of a complicated process. It appears that there may be a fast initial step in the aggregation process which is *p*H-reversible but that a subsequent slow reaction stabilizes the aggregate (formation of intermolecular disulfide bonds through thiol-disulfide exchange).[26]

(25) P. Bro, S. J. Singer and J. M. Sturtevant, THIS JOURNAL, **77**, 4924 (1954).

(26) A serious problem that arises in this interpretation is the well-known fact that thiol–disulfide exchange reactions are normally vanishingly slow in mildly acidic solution. This problem has been discussed recently by Jensen[27] who suggests alternative ways in which the sulfhydryl group might be involved in the dimerization reaction. The essential point to be made here is that whatever the nature of this reaction, it is slow and is preceded by a rapid reversible dimerization.

(27) E. V. Jensen, *Science*, **130**, 1319 (1959).

Doubtless the most interesting and characteristic feature of the reaction is the sharp *p*H optimum. The fact that this optimum occurs well below the isoelectric *p*H (approximately 4.3 under the conditions of these experiments) strongly suggests that some structural rearrangement of the protein must precede aggregation. On the basis of simple electrostatic effects the optimum should fall at or rather close to the isoelectric *p*H. It is possible to suggest a reasonable explanation for the *p*H optimum in terms of other available information on the structural alterations which take place in this protein in acid solution.

Viscosity,[4,5] optical rotation[4] and sedimentation[18,28] experiments have been interpreted as indicating that a modification of the molecular structure of BPA occurs at *p*H below *ca.* 3.5 and it is in this *p*H region that the percentage of dimer begins to increase abruptly. Although some degree of molecular expansion or unfolding may be an essential prerequisite to dimerization, an alternative explanation is equally, if not more, plausible. It seems possible that dimerization occurs readily only when native BPA has been converted to a more "aggregatable" isomeric form[29–33] and that expansion is incidental in that it also occurs as a consequence of this conversion.[5] Evidence in favor of this alternate explanation is found in Foster and Aoki's[32] theoretical treatment of the isomerization process. As shown in Fig. 5 of their paper the concentration of their final isomeric "F" form is maximal near *p*H 3.3. At *p*H values lower than 3.3 molecular expansion reduces the concentration of this form and hence expansion should compete with dimerization, both processes tending to reduce the concentration of the F form. If the tendency of BPA to aggregate is proportional to the concentration of the F form, then one would predict that the percentage of dimer in the present investigation should be maximal near *p*H 3.3. Figure 3 illustrates that such is the case.

Warner and Levy[34] in heat denaturation studies with BPA have observed a relationship between the rate of heat denaturation and *p*H which is also remarkably similar to the per cent. dimer *vs.* *p*H curve in Fig. 2. The maximum of their curve is at *p*H 4 (at 56°) but is shifted to a lower *p*H when the temperature is lowered.[35,36] This suggests a basic similarity between heat denaturation and the low *p*H dimerization. This similarity is bolstered by a study of Steinrauf and Dandliker[37] who concluded that heat denaturation also involves two distinct steps such as suggested above, namely a rapid reversible step followed by a slow irreversible disulfide exchange.

Rachinsky and Foster[38] have given evidence that the isomerized (F) form of plasma albumin is

(28) M. J. Kronman and J. F. Foster, *Arch. Biochem. and Biophys.*, **72**, 205 (1957).

(29) K. Aoki and J. F. Foster, THIS JOURNAL, **79**, 3385 (1957).

(30) K. Aoki and J. F. Foster, *ibid.*, **79**, 3393 (1957).

(31) J. F. Foster and K. Aoki, *ibid.*, **80**, 1117 (1958).

(32) J. F. Foster and K. Aoki, *J. Phys. Chem.*, **61**, 1369 (1957).

(33) J. F. Foster and K. Aoki, THIS JOURNAL, **80**, 5215 (1958).

(34) R. Warner and M. Levy, *ibid.*, **80**, 5735 (1958).

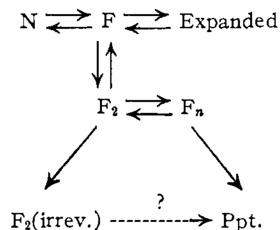
(35) R. Warner and M. Levy, *J. Phys. Chem.*, **58**, 106 (1954).

(36) R. J. Gibbs, *Arch. Biochem. Biophys.*, **52**, 340 (1954).

(37) K. Steinrauf and W. B. Dandliker, THIS JOURNAL, **80**, 3833 (1958).

readily salted-out of solution. Cann[39] has also shown that plasma albumin is readily precipitated in acid solution in presence of 0.15 *M* perchlorate. He found a rapid reversible precipitation with a sharp *p*H optimum similar to that in Fig. 3, followed by a slow irreversible precipitation. He considered the rapid precipitation to involve the isomeric form of the protein and the slow precipitation to procede through the expanded form. It appears that his results could equally well be explained through a slow reaction proceeding through the F form.

It is suggested that all of the phenomena discussed above can be explained through a single reaction pattern as illustrated in the reaction diagram, where F_n is a soluble polymer or reversible precipitate, depending on conditions. At room temperature and low ionic strength (for example 0.02 *M* chloride) the expansion equilibrium is favored and



the activity of F form might never be high enough to result in aggregation. At higher ionic strength expansion is strongly repressed[4] and the dimerization equilibrium would be favored, with subsequent slow formation of irreversible dimer. At very high ionic strength (in chloride) or moderately high ionic strength in presence of strongly bound anions such as perchlorate expansion is almost completely repressed and formation of higher aggregates, both reversible and irreversible, favored due to increased screening and reduction of the net positive charge on the protein. At elevated temperatures, as in heat denaturation, all processes are presumably speeded, especially the slow irreversible step.

(38) M. R. Rachinsky and J. F. Foster, *Arch. Biochem. Biophys.*, **70**, 283 (1957).

(39) J. Cann, *J. Phys. Chem.*, **63**, 1545 (1959).

[CONTRIBUTION FROM THE CHEMISTRY DEPARTMENT AND OCEANOGRAPHIC INSTITUTE OF THE FLORIDA STATE UNIVERSITY TALLAHASSEE, FLA.]

The Thermal Copolymerization of Amino Acids Common to Protein[1]

BY SIDNEY W. FOX AND KAORU HARADA

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By use of an excess of the dicarboxylic amino acids, the dry, eighteen amino acids common to proteins can be simultaneously copolymerized in a few hours at temperatures above 150°. Changes in the constitution of the polymers are described for variations in the conditions of reaction. The properties of the synthetic products are compared with known properties of natural proteins.

Attempts to synthesize protein under hypothetically primitive terrestrial conditions[2] have yielded anhydropolymers which contain all of the eighteen amino acids common to protein and which otherwise resemble protein in many of their properties.[3] (Such materials are referred to as *proteinoids*.) The critical conditions are the maintenance of a hypohydrous state, as by a temperature of above 100°, and the employment of a sufficient excess of dicarboxylic amino acid. The decomposition ordinarily encountered during uncontrolled heating of amino acids above 150° results particularly from the neutral amino acids in the absence of dicarboxylic amino acid.[4] In thermal copolymerizations, the protective effect of excess dicarboxylic amino acid is lost to a significant degree when the temperature exceeds 210°.[5]

One salient feature of the thermal copolymerizations is the finding that the order of amino acid residues in the polymeric products is not completely random, as judged by comparison of total composition and N-terminal composition. Such an effect was first observed most definitively in the pyrocondensation of aspartic acid and glutamic acid.[5] It is of particular interest to compare such effects when the sixteen other amino acids are copolymerized simultaneously ("panpolymerized") with an excess of the dicarboxylic amino acids, inasmuch as such studies may help to explain sequences in natural proteins. Comparisons of this type, similar studies of total amino acid composition, and the effect of other factors on the yield and chemical nature of the products are reported in this paper.

In addition, data presented earlier and data in this paper are reviewed in comparing the synthetic product with natural protein.

Experimental

Proportions of Reactants.—The proportions of all sixteen amino acids exclusive of the dicarboxylic amino acids were the same in all experiments, 0.03 mole each of DL-alanine, L-arginine monohydrochloride, L-cystine, glycine, L-histidine hydrochloride monohydrate, DL-isoleucine, DL-leucine, L-lysine monohydrochloride, DL-methionine, DL-phenylalanine, L-proline, DL-serine, DL-threonine, DL-tryptophan, DL-tyrosine and DL-valine. This mass was intimately ground to a fine powder. Mixture A consisted of 10 g. of L-glutamic acid, 10 g. of DL-aspartic acid and

(1) Presented in part at the 135th Meeting of the American Chemical Society at Boston on April 9, 1959. This research is supported by Grant G-4566 of the National Science Foundation, by the General Foods Corporation, and by Grant C-3971 of the National Institutes of Health, U. S. Public Health Service. Contribution No. 138 of the Oceanographic Institute of The Florida State University.

(2) S. W. Fox and M. Middlebrook, *Federation Proc.*, **13**, 211 (1954).

(3) S. W. Fox and K. Harada, *Science*, **128**, 1214 (1958); S. W. Fox, K. Harada and A. Vegotsky, *Experientia*, **15**, 81 (1959).

(4) S. W. Fox and G. D. Maier, General Petroleum Geochemistry Symposium, Fifth World Petroleum Congress, June 4, 1959, p. 9.

(5) K. Harada and S. W. Fox, *Arch. Biochem. Biophys.*, **86**, 274 (1960).