# The Microheterogeneity of Plasma Albumins. V. Permutations in Disulfide Pairings as a Probable Source of Microheterogeneity in Bovine Albumin*

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ABSTRACT: The microheterogeneity of crystallized bovine plasma albumin, as determined by the previously described solubility–pH profile method, has been found to increase on storage of solutions. Under similar conditions, the observed broadening of the population is much greater for charcoal-defatted protein than for non-defatted or acid-defatted preparations. The rate of the reaction increases markedly with increasing pH over the range 7–10 but is significant, in charcoal-defatted proteins, at the isoionic pH. Owing to the observation that blocking of the sulfhydryl group by iodoacetamide or *N*-ethylmaleimide virtually eliminates the reaction, broadening is attributed to sulfhydryl-catalyzed disulfide interchange. No detectable increase in dimer or polymer content accompanies the process; hence interchange must be almost exclusively intramolecular. The rate of broadening increases with increasing temperature and is reduced by increasing ionic strength. The results are discussed in light of published information on the reducibility of disulfide bonds and hydrogen–deuterium exchange of the protein as influenced by the various parameters mentioned above and it is concluded that the interchange reaction probably involves relatively open conformational isomers which are in equilibrium with the native protein. The possibility that such disulfide interchange might take place under physiological conditions is considered and it is suggested that at least some of the observed microheterogeneity in crystallized bovine plasma albumin may arise from imperfect disulfide pairing.

It seems clear that there must be a number of disulfide configurations accessible to the bovine plasma albumin molecule and the distribution of pairings actually assumed may depend to some extent upon environmental conditions, especially on bound ligands such as fatty acids.

We have interpreted the observed behavior of plasma albumin with respect to the N–F transition and associated phenomena on the basis of microheterogeneity (Sogami and Foster, 1963; Foster *et al.*, 1965). According to this proposal the protein consists of a population of many different but closely related species. Various species undergo the N–F transition at slightly different characteristic values of pH, and the observed N–F transition of the whole population is the resultant of a population distribution function superimposed on the very sharp pH dependence of the transitions of the individual species. The transition from N to F form of a given species is very fast, while interconversion of different species is immeasurably slow under the conditions of the N–F isomerization.

It has been possible to prepare subfractions (Petersen and Foster, 1965a) of plasma albumin by taking advantage of the very low solubility of the F form in concentrated salt solution. Such subpopulations differ in the pH range over which they undergo the N–F transitions as was shown by solubility studies (Petersen and Foster, 1965a) and by optical rotation measurements at 313-mμ (Petersen and Foster, 1965b). Differences between subfractions have also been shown by other techniques such as ultraviolet difference spectrophotometry, hydrogen ion titration, and heat denaturation but no significant differences were observed in optical rotatory dispersion behavior (Petersen and Foster, 1965b). In addition, the tyrosyl chromophores in the more stable species are, on the average, more perturbed by the protein fabric than are tyrosyl residues in less stable species (Petersen and Foster, 1965b). This is true when the protein is in the N state, but when all species are in an expanded state the chromophores exist in essentially equivalent environments.

Štokrová and Šponár (1963) suggested independently that plasma albumins consist of populations of molecules differing in their heat lability and we have shown a parallel to exist between stability toward the N–F transition and heat denaturation. Thus, the microheterogeneity probably involves variations in general stability of the native form of the protein molecules.

The structural basis for this microheterogeneity remains unresolved. Unquestionably, part of the variabil-

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ity resides in the amount and kind of bound lipophilic impurities (Sogami and Foster, 1967; McMenamy and Lee, 1967) but there are almost certainly some as yet undefined intrinsic contributions to the microheterogeneity as well (Sogami and Foster, 1968; Wong and Foster, 1968). Among several conceivable covalent contributions we have suggested (Foster *et al.*, 1965), one which merits special consideration is the possibility of variations in pairing of half-cystine residues through disulfide bonding. Since this protein contains 17 or 18 such disulfide linkages and a single unpaired cysteine the possible number of pairings is very great. On the other hand, as we have pointed out, the fact that the microheterogeneity exists in crystallized samples must severely restrict the possible number of such pairings. We reported earlier (Petersen and Foster, 1965c) that the population of molecules is broadened under conditions favorable to SH-catalyzed disulfide interchange implying that fluctuations in pairing of the half-cystine residues might be a contributing factor, but those results have not previously been described in detail. Recently, we have observed (Sogami and Foster, 1967, 1968) that plasma albumin samples more thoroughly defatted by the charcoal procedure of Chen (1967) are much more labile than those used in the earlier experiments, which were defatted by a more drastic but less effective acid treatment. The purpose of this paper is to report both the earlier experiments and recent ones using charcoal-defatted protein and to show that plasma albumins are indeed extremely prone to intramolecular disulfide-interchange reactions which markedly broaden the population.

## Experimental Section

*Albumin Preparations.* The experiments described here extended over a period of nearly 4 years and employed several different albumin samples pretreated in differing ways. Some of the earlier experiments were conducted with crystallized bovine plasma albumin lot 9 obtained from Pentex, Inc. This sample contained approximately 7% dimer as judged by velocity ultracentrifugation and appeared to be virtually free of lipid contaminants. Since deionization (Dintzis, 1952) of the protein had essentially no effect on the observed results, this sample was used without further purification except for filtration through Millipore filters (0.45 mμ). Protein samples referred to as "conventionally defatted" were prepared from crystallized bovine plasma albumin obtained from Armour Pharmaceutical Co., either lot W69204 or lot B70411. Such samples were defatted by low pH treatment followed by filtration through a Millipore filter (Williams and Foster, 1959). After defatting they were deionized by the procedure of Dintzis (1952), lyophilized, and stored as the dry powder in the cold room. In latter experiments charcoal-defatted protein was prepared by a slight modification of the method of Chen (1967) as described by Sogami and Foster (1968). These protein samples were not lyophilized but were stored in the cold room (*ca.* 2°) in the isoionic state (pH 5.4–5.5) in the presence of 0.03–0.05 M KCl to protect against ageing effects (Sogami and Foster, 1968). The solutions were deionized by passing through a Dintzis column (Dintzis, 1952) as rapidly as possible immediately before starting an experiment.

Table I summarizes information on dimer content and SH analyses of the various albumin preparations employed.

Bovine nonmercaptalbumin was prepared by permitting interchange to occur with cystine (Andersson, 1966; Isles and Jocelyn, 1963). Cystine (5 moles/mole of protein) was added to bovine plasma albumin (conventionally defatted lot W69204) solution in 0.10 Γ/2 Tris buffer of pH 8.01, stirred gently for 10 hr at 25°, and then permitted to stand in a cold room for 1 week. The solution was filtered through a Millipore filter, dialyzed exhaustively against several changes of deionized water, and lyophilized. This modification was applied only to conventionally defatted albumin since charcoal-defatted albumin is unstable under the conditions employed. Iodoacetamide-blocked charcoal-defatted bovine plasma albumin was prepared by adding 3 moles of iodoacetamide (Sigma Chem. Co.) per mole of protein to a bovine plasma albumin solution of pH 7.00 in 0.035 M KCl and permitting the solution to stand in the cold room for approximately 24 hr after which the solution was exhaustively dialyzed. In earlier experiments with nontreated bovine plasma albumin lot 9, blocking was performed by adding 5 moles of either iodoacetamide or *N*-ethylmaleimide (Nutritional Biochemical Corp.) per mole of protein and storing the solution at pH 7.0 for 24 hr in the cold room.

*Determination of Solubility–pH Profiles.* The method employed for assessing alterations in microheterogeneity was determination of the solubility–pH profile in 3.0 M KCl. The early experiments were conducted using the previously described (Petersen and Foster, 1965a) continuous titration method employing filter sticks for isolating aliquots of supernatant at various pH values. In later studies the equilibration method described by Sogami and Foster (1968) was used. With this method, it is possible to employ protein concentrations of approximately 0.1% (as compared with 0.75% by the older method); the results are more reproducible and certain uncertainties such as adsorption on the filter and kinetic effects are minimized or eliminated (Sogami and Foster, 1968).

*Analytical Procedures.* For determination of free SH groups, *p*-hydroxymercuribenzoate (sodium salt, Sigma Chemical Co.) was employed in 0.317 M acetate buffer (pH 4.63) following the procedure of Benesch and Benesch (1962). Titrations were carried out using a microburet of 0.250-ml capacity (L. S. Starrett Co.). Later experiments employed dithiobis(2-nitrobenzoic acid) (Aldrich Chemical Co.) in pH 8.00 phosphate buffer (0.10 Γ/2) by the procedure of Ellman (1959). It was found necessary to control the pH closely at 8.00 in order to obtain reproducible results.

The dimer content of albumin preparations was estimated from sedimentation velocity patterns carried out in the Spinco Model E ultracentrifuge, typically at 23° and 59,780 rpm. Kel-F centerpieces were employed and patterns were recorded with the phase-plate schlieren optical system.

Optical rotation and optical rotatory dispersion mea-

TABLE I: Sulfhydryl and Dimer Contents of Various Protein Preparations.[a]

| | SH Content (mol/mol) | Dimer Content (%) |
|---|---|---|
| Nontreated bovine plasma albumin (lot W 69204) | 0.63 | |
| Conventionally defatted deionized bovine plasma albumin (lot W 69204) | 0.61 | 5.4 |
| Deionized bovine plasma albumin (lot W 69204) | 0.54 | 10.0 (polymers 1%) |
| Nontreated bovine plasma albumin (A 70011) | 0.75 | |
| Nontreated bovine plasma albumin (B 70411) | 0.74 | |
| Charcoal-defatted deionized bovine plasma albumin (B 70411) | 0.73[b] | 3.3 |
| Charcoal-defatted deionized bovine plasma albumin (B 70411) aged in deionized state approximately 2 weeks, 2° | 0.70[b] | 5.0 |
| Iodoacetamide-blocked charcoal-defatted deionized bovine plasma albumin (B 70411) | 0.1[b] | |
| Bovine nonmercaptalbumin (W 69204) | 0 | 5.6 |

[a] SH titer was calculated assuming a molecular weight of 66,000. [b] By dithiobis(2-nitrobenzoic acid) analysis. Other data were by *p*-mercuribenzoate titration.

surements were conducted with a Bendix-Ericsson Polarmatic Model 460C recording spectropolarimeter. The conditions and precautions taken were as described by Sogami and Foster (1968). All measurements were at ambient temperature (24 ± 1°) and the data were analyzed by means of the equations of Moffitt and Yang (1956) and of Shechter and Blout (1964), assuming a mean residue weight of 118.

*Miscellaneous.* Protein concentrations were determined by absorbance in a Hitachi:Perkin-Elmer 139 spectrophotometer assuming $E^{1\%}_{1\,cm}$ to be 6.67 at 279 mμ. All water employed was distilled, passed through a deionizer (Barnstead Bantam Model BD-1), and had a specific resistance greater than $10^6$ ohms. Cellophane tubing (Visking) was pretreated by boiling in 50% saturated $NaHCO_3$ followed by copious washing with deionized water. All chemicals employed, other than those specifically mentioned above, were of reagent grade.

*pH Measurements.* In the early solubility determinations, a Radiometer type 22 pH meter equipped with G 22C glass electrode and open-reference electrode K 130 was employed. In later experiments, a Radiometer type 25 instrument equipped with expanded scale was employed using in some cases a Beckman general-purpose glass electrode (41263) and a Beckman frit-junction reference electrode and in other cases a Radiometer combined electrode GK2026C. In the case of either the frit-junction electrode or the combined electrode, it was found necessary to replace the electrodes frequently, as described previously (Sogami and Foster, 1968). The pH meters were standardized against Sargent buffers of both pH 4.01 and 7.00. With the precautions we have described it was possible to reproduce pH–solubility profiles to within ±0.005 pH unit, the main source of error being errors arising in the pH measurement in the high-salt system.

## Results and Discussion

*Blocking of the SH Groups.* Results of the SH analyses on various bovine plasma albumin preparations are summarized in Table I. These values were calculated on the basis of a molecular weight of 66,000 and are in reasonable agreement with most published values when these are recalculated to the same basis. One exception is the value of 0.85 mol of SH/mol reported by Boyer (1954), which value may be unrealistically high due to cleavage of the mixed disulfide bond of nonmercaptalbumin during the prolonged reaction (Boyer, 1960).

Since the early experiments on the ageing phenomenon pointed to the probable involvement of the free SH group as a catalyst of the disulfide-interchange reaction, numerous ageing experiments have been performed using bovine plasma albumin with the SH group blocked. Ideally, for such purposes the blocking reagent should completely mask all of the SH groups without any further modification of the protein. Since no masking reagent can be expected to be absolutely specific, concern is raised as to the possibility that derivatization might enhance the microheterogeneity due to modification of different protein molecules in different ways and to differing extents. It is therefore of interest and importance that, with any of the blocking agents employed, it has been possible to block virtually all of the SH groups with no detectable broadening of the solubility–pH profile. The iodoacetamide-blocked charcoal-defatted protein showed approximately 0.1 mol/mol of reactive SH but the protein blocked by exchange with cystine (synthetic bovine nonmercaptalbumin) showed

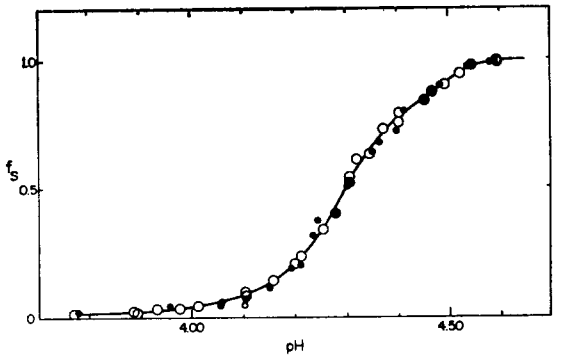


FIGURE 1: Solubility–pH profiles in 3.0 M KCl as determined by the equilibration method of Sogami and Foster (1968). (○) Conventionally defatted bovine nonmercaptalbumin lot W 69204; (●) conventionally defatted bovine nonmercaptalbumin prepared from bovine plasma albumin lot W 69204.

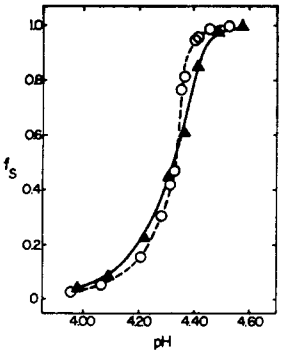


FIGURE 2: Solubility–pH profiles as determined by the continuous titration method for bovine plasma albumin before storage (circles) and after storage at pH 8.5 and 2° for 100 hr (triangles).

no measurable reaction with *p*-hydroxymercuribenzoate (Table I). The fact that this latter derivatization does not enhance the microheterogeneity is shown in Figure 1 which compares the solubility–pH profiles of bovine plasma albumin and bovine nonmercaptalbumin. Similar results have been obtained repeatedly with iodoacetamide- and *N*-ethylmaleimide-blocked protein.

*Population Broadening and Its Dependence upon pH.* A fundamental assumption in the microheterogeneity model is that the width of the pH range in which a sample undergoes the N–F transformation directly reflects the width of its population distribution. In the present study, wherein solubility in 3.0 M KCl is used to detect the N–F transition, it is assumed that sample A is more heterogeneous than sample B if the solubility–pH profile of A covers a wider pH range than the profile of B. In specifying this pH range, it is convenient to define a quantity $\Delta pH_{10}^{90}$. This is the amount by which the pH must be lowered to proceed from a condition in which 90% of the protein is in solution to one in which 10% is in solution. The choice of the arbitrary limits is based on the general shape of the solubility curves. The error in ΔpH is minimized by considering only the relatively linear central portion of a curve.

An example of broadening of the population which occurs on ageing solutions of bovine plasma albumin under certain conditions is shown in Figure 2. In this case a solution of nondefatted bovine plasma albumin (lot 9) was stored at 2° and pH 8.5 for 100 hr. To a first approximation, the ageing results in a broadening of the solubility–pH profile with little shift in the pH of the midpoint. However, at higher pH the effect is more pronounced and a general shift of the solubility–pH curves toward higher pH is observed. With charcoal-defatted bovine plasma albumin the effect is much more marked than with nondefatted bovine plasma albumin and in all cases it is clear that there is a general shift of the solubility profiles toward higher pH rather than a symmetrical broadening. Figure 3 summarizes results on charcoal-defatted protein at various pH values. These protein samples were aged at approximately 2% protein with no added salt and the pH was adjusted to the values indicated by adding 0.10 M KOH.

Clearly the degree of population broadening under these conditions increases with an increase of the pH at which the solution is aged. Figure 4 summarizes results obtained at various pH values with the various bovine plasma albumin preparations and derivatives. The results are plotted here as $\delta(\Delta pH_{10}^{90})$ *vs.* the pH at which the sample was aged. In these experiments no salt was added and in all cases but two the samples were aged at 2° for 83–100 hr. Two samples, as indicated in the caption to Figure 4, were aged at 25° for 23 hr. With both the conventionally defatted protein (and the nondefatted Pentex protein which is very similar) and the charcoal-defatted protein the pH dependence suggests the involvement of a prototropic group of p*K* approximately 8.0. Since SH-catalyzed disulfide interchange evidently proceeds *via* the ionized mercaptide group (Cecil and McPhee, 1959) this pH dependence strongly suggests the possibility that this is the reaction responsible for the observed population broadening.

*Evidence for Intramolecular Disulfide Interchange.* To test the possible role of disulfide interchange in the population broadening, ageing experiments were conducted with SH-blocked bovine plasma albumin. The results of these experiments are also shown in Figure 4. In the early experiments with conventionally defatted or nondefatted bovine plasma albumin no attempt was made to remove the excess blocking reagent. The reagent (iodoacetamide or *N*-ethylmaleimide) was simply added to the bovine plasma albumin solution and the solution was stored at pH 7.00 and 2° for 24 hr. The pH was then raised to the desired value and the solution permitted to age. Clearly, either iodoacetamide or *N*-ethylmaleimide blocking almost completely eliminates the population broadening in nondefatted or conventionally defatted protein. The experiments on iodoacetamide-blocked charcoal-defatted protein were conducted by removing (by dialysis) the excess reagent prior to conducting the ageing experiments. Also a ratio of only 3 mol of iodoacetamide/mol of bovine plasma albumin was employed in this case rather than 5 in the earlier experiments. This iodoacetamide-blocked derivative retained approximately 0.1 mol of reactive SH/mol of protein. The rate of population broadening in this case is seen in Figure 4 to be significant but greatly reduced

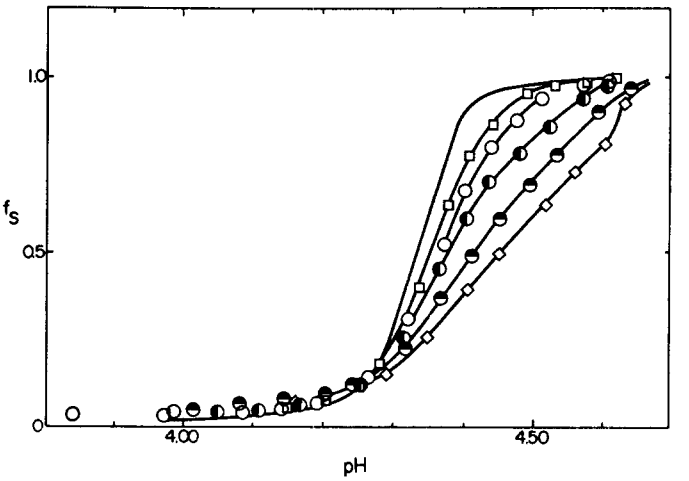


FIGURE 3: Solubility–pH profiles in 3.0 M KCl as determined by the equilibration method for charcoal-defatted bovine plasma albumin lot B 70411 (the control) and various aged samples were prepared from this same protein. The solid curve without experimental points is the control. The aged samples are (□) isoionic (pH *ca.* 5.5), (○) pH 7.07, (◐) pH 7.521, (◓) pH 8.02, and (◇) pH 8.51. Samples, were aged in salt-free solution for 83 hr except for the isoionic sample which was aged for 109 hr.

as compared with the unblocked control. The sample of cysteine-blocked bovine plasma albumin aged at pH 9.97 for 76 hr showed no detectable broadening of the population.

In one experiment with charcoal-defatted bovine plasma albumin the SH titer as determined by the dithiobis(2-nitrobenzoic acid) method showed virtually no decrease after ageing for 2 weeks in the deionized state (Table I). Simpson and Saroff (1958) observed a half-time of approximately 40 days for the decrease of reactive SH groups in human serum albumin even at pH 10.5. We conclude that the reaction responsible for population broadening is, in the main, disulfide interchange catalyzed by the mercaptide group and that this reaction proceeds with no net loss of the latter groups.

It might be anticipated that disulfide interchange would lead to some formation of intermolecular disulfide bonds with resultant formation of dimeric or polymeric species. This possibility was checked carefully by ultracentrifugation and no evidence for any increase in dimer or polymer content was seen. Two typical examples of such sedimentation analyses are shown in Figure 5. One of the examples shown involved nondefatted protein aged at pH 9.5 for 93 hr, and the other patterns are of charcoal-defatted bovine plasma albumin aged under two sets of conditions. The solubility profiles of the latter two samples differed significantly from the control as shown in Figures 3 and 6.

*Effect of Ionic Strength on the Population Broadening.* Results assembled in Figure 6 demonstrate the degree of population broadening to be markedly influenced by the ionic strength of the solution. At pH 7.45 ± 0.01 the effect is nil at ionic strength 0.20, small but measurable at 0.05–0.10, and marked at essentially zero ionic strength (no added salt). Even at the isoionic pH (*ca.* 5.4) substantial broadening is seen in the absence of salt. This effect has been mentioned previously (Sogami and Foster, 1968).

*Changes in pH Accompanying Population Broadening.* The bovine plasma albumin solutions subjected to the ageing conditions were in all cases unbuffered except for the protein *per se*. It was observed that the pH of the solutions decreased slowly during the ageing process. For example, the pH of a solution of bovine plasma albumin lot 9 initially at 9.5 decreased to 9.1 in 24 hr even though the tube was sealed under nitrogen. From the titration curve of bovine plasma albumin, as well as from preliminary pH-Stat experiments, this decrease in pH was estimated to correspond to approximately 3.0 mol of base consumed/mol of protein. Partial deamidation of the protein as observed by Flatmark (1966) for cytochrome *c* was considered as a possible explanation for this decrease in pH. In several cases, the amount of ammonia liberated was measured by conducting the ageing experiment (bovine plasma albumin lot 9) in a modified Conway dish and absorbing the ammonia in dilute $H_3BO_3$. Over the pH range 8.5–10.5 the ammonia liberated corresponded to only approximately 0.1–0.2 mol/mol of protein in 48 hr. It would appear that deamidation cannot be the major contributing factor.

Figure 7 presents the degree of population broadening

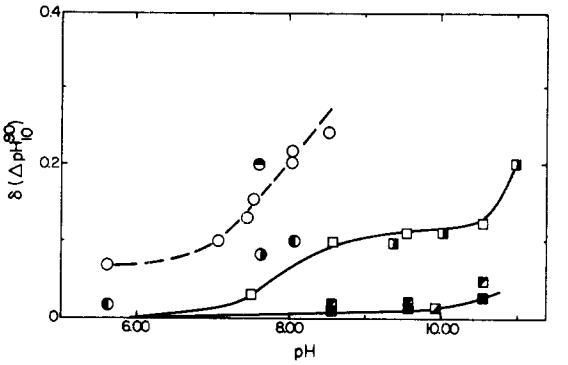


FIGURE 4: Increase in microheterogeneity of bovine plasma albumin during storage as indicated by the change in $\Delta pH^{80}_{10}$ of the solubility–pH profile. All points indicated by square symbols denote experiments with either nondefatted bovine plasma albumin (lot 9) or conventionally defatted deionized bovine plasma albumin (lot W 69204) while circular symbols refer to experiments with charcoal-defatted deionized bovine plasma albumin (lot B 70411). (□) 100 hr at 2°; (◨) 76–88 hr at 2°; (■) iodoacetamide-blocked bovine plasma albumin, 100 hr at 2°; (◩) *N*-ethylmaleimide-blocked bovine plasma albumin, 100 hr at 2°; (◪) bovine nonmercaptalbumin, 76 hr at 2°; (○) 83 hr at 2°; (◓) 23 hr at 25°; (◐) iodoacetamide-blocked charcoal-defatted bovine plasma albumin with 0.1 mol/mol of residual SH group, 83 hr at 2°; (◑) the same iodoacetamide-blocked protein, 23 hr at 25°.

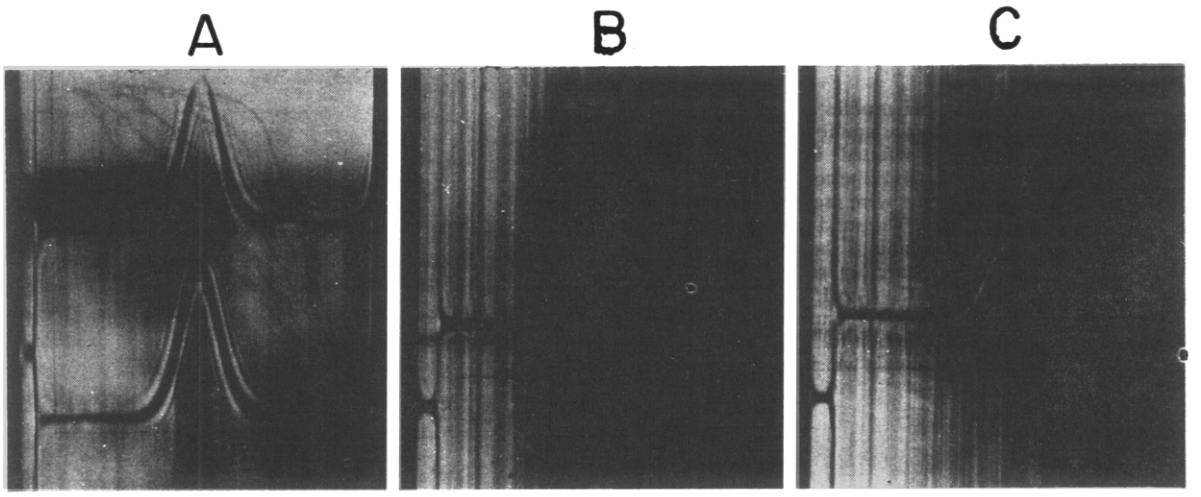


FIGURE 5: Sedimentation velocity patterns on control and aged bovine plasma albumin preparations. Ultracentrifuge runs were at 59,780 rpm and isoionic pH (*ca.* 5.5) and photographs were taken after 90-min sedimentation. Pattern A is a two-cell run with nondefatted lot 9 bovine plasma albumin (control, upper pattern) and the same protein after storage at pH 9.50, 2° for 93 hr (lower pattern). Ultracentrifuge runs were in the presence of 0.05 M NaCl and while both samples contain some dimer (small peak) there is no significant increase in this component on ageing. Patterns B and C were obtained with charcoal-defatted lot B 70411 bovine plasma albumin and were run in the presence of 0.10 M KCl. Pattern B corresponds to a sample stored 72 hr in salt-free solution at pH 7.47 and 2°. Pattern C was obtained with the same protein stored at the isoionic pH (deionized) for approximately 2 weeks. Neither pattern indicates the presence of a significant amount of dimer but the solubility–pH profiles of these two samples differ significantly from the control as shown in Figures 3 and 6.

in a number of experiments as a function of the observed drop in solution pH. No direct correlation exists between the pH of ageing and the pH decrease. For example, the bovine nonmercaptalbumin sample aged at pH near 10 showed almost no broadening of the population and virtually no decrease in pH. It is difficult to avoid the conclusion that the pH drop is directly related in some way to the degree of broadening of the population. The results in Figure 7 suggest, further, that two different broadening reactions are involved corresponding to the two linear regions of curve A. The first of these processes results in substantial population broadening with very little shift in solution pH. The points shown by squares in Figure 7 (curve B) were obtained with a sample of charcoal-defatted protein which was first permitted to age at the isoionic pH until $\Delta pH_{10}^{90}$ had increased from 0.17 to 0.38 and then was aged under the conditions indicated. The decrease in pH measured and shown in Figure 7 is that in the second ageing phase only. In this case the data yield a nearly linear relation between the pH decrease and degree of broadening, the slope corresponding to the second phase in curve A. We would suggest that much of the pH decrease results from a subtle modification of the p*K* values of some of the protropic groups which are buffering in the pH range of the experiments (7–10). This might result from a simple relax-

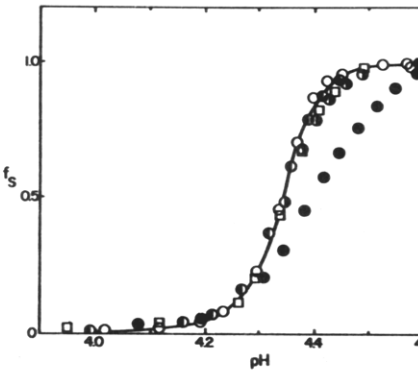


FIGURE 6: Effect of ionic strength on population broadening. The solid curve, experimental points omitted, represents the unaged control, charcoal-defatted and deionized bovine plasma albumin lot B 70411. Experimental points represent results on samples aged for 72 hr at 2° and pH 7.45 ± 0.01, with varying concentrations of KCl. (○) 0.20 M KCl; (◐) 0.15 M; (□) 0.10 M; (◑) 0.05 M; (●) no added salt.

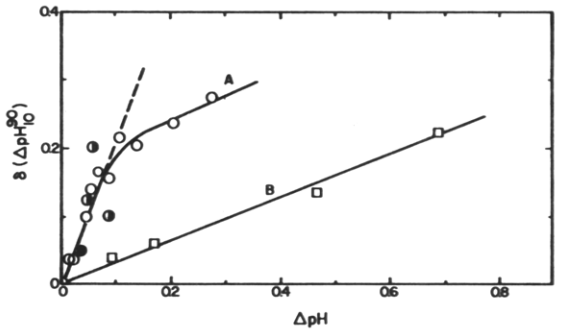


FIGURE 7: Relation between the degree of population broadening on ageing, $\delta(\Delta pH_{10}^{90})$, and the observed decrease in solution pH, ΔpH. Curve A: (○) Charcoal-defatted bovine plasma albumin, corresponding to some of the experiments in Figures 3 and 6; (◑) three experiments on conventionally defatted bovine plasma albumin aged at pH 9.4, 10.0, and 11.0 in absence of salt; (●) a single experiment on bovine nonmercaptalbumin aged at pH 10.0. Curve B and the squares correspond to experiments on charcoal-defatted bovine plasma albumin which had been aged previously in isoionic deionized solution for 10 days at 2°. The four samples shown (from left to right) were aged for 72 hr and 2° at pH values of 7.7, 8.1, 9.1, and 9.7 in salt-free solution.

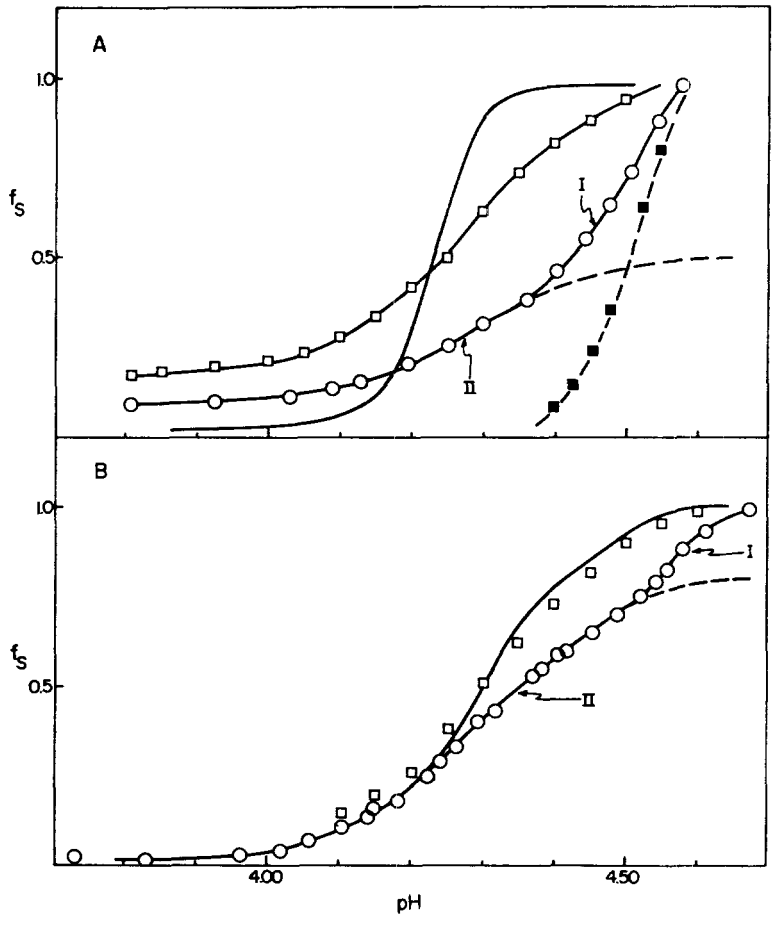


FIGURE 8: Resolution of the solubility–pH profiles for aged bovine plasma albumin in terms of two hypothetical components. In A, the solid line is the profile for the control protein, charcoal-defatted bovine plasma albumin; open circles are for the protein aged at pH 9.7 without added salt for 72 hr at 2° after prior ageing for *ca.* 10 days at 2° in the isoionic state; filled squares, hypothetical component I; open squares, hypothetical component II. Curves B, in which the same symbolism is followed, represent a similar experiment with conventionally defatted bovine plasma albumin aged at pH 10.0 for 85 hr at 2°.

ation of the coulombic repulsion due to the disulfide interchange. Thus, since the net charge on the protein is negative in this range, it is postulated that disulfide-interchange results in a migration toward configurations having a reduced intramolecular electrostatic repulsion, hence a reduced electrostatic potential. This would tend to produce some net loss of protons from the protein with an accompanying reduction in solution pH.

*On the Role of Bound Impurities.* The data presented above, especially in Figure 6, show the charcoal-defatted protein to be much more labile toward the postulated disulfide-interchange reaction than either conventionally defatted protein or the nondefatted Pentex lot 9 protein. Sogami and Foster (1968) observed a much greater lability of charcoal-defatted protein in the isoionic and acid pH ranges. We must not lose site of the fact that the population distribution of the charcoal-defatted protein is initially much narrower than that of the other samples so that small enhancement of the breadth of the solubility–pH profile is more readily detected and is more spectacular. Nevertheless, it seems clear that bound impurities, presumably fatty acids (Chen, 1967), exert a stabilizing action with respect to the disulfide-interchange reaction.

Previously, attention was focused on changes in the breadth of the solubility–pH profiles in terms of $\Delta pH_{10}^{90}$ without regard to the detailed shape of the profiles. Figure 8 shows that extreme broadening gives rise to profiles which are inflected and give the impression of a bimodal distribution of species. In Figure 8A, conducted with charcoal-defatted bovine plasma albumin, the broad curve after ageing (shown by circles) has been somewhat arbitrarily subdivided into two components of equal amount and the curves for these postulated components are shown by the open and filled squares. To the extent to which this analysis is meaningful, one of these components (labeled component II) shows a symmetrical broadening about the initial midpoint pH; the other, component I, shows a strong shift toward higher pH with virtually no broadening. When the same analysis is carried out for aged conventionally defatted bovine plasma albumin (Figure 8B) the amount of component II must be considered to be much greater (*ca.* 80%) and it scarcely broadens at all. This result suggests that most of the protein in this sample did not participate in the broadening reaction, in contrast to the result with charcoal-defatted protein.

*Changes in Folding Accompanying Population Broadening.* Table II summarizes results of some optical rotation and optical rotatory dispersion measurements of charcoal-defatted bovine plasma albumin before and after ageing at pH 7.6 and 8.1. The helix content of the protein, as judged by any of the methods (depth of the first Cotton trough at 233 mμ and analysis of the dispersion curves by either the $b_0$ method of Moffitt and Yang (1956) or the two-term Drude equation of

TABLE II: Summary of Optical Rotatory Dispersion Results on Control and Aged Bovine Plasma Albumin.[a]

| Protein | Helix Content (%) from: $[M']_{233}$[b] | $b_0$[c] | $H_{225}$[d] | $H_{193}$[d] | Cotton Effect around 290 mμ |
|---|---|---|---|---|---|
| Control, charcoal-defatted bovine plasma albumin | 51.2 | 53.2–54.4 | 54.3 | 51.2 | (++) |
| Aged at pH 7.6, 2° for 86 hr | 50.1 | 54.4–55.5 | 53.1 | 49.5 | (±) |
| Aged at pH 8.1, 2° for 86 hr | 49.7 | 53.2 | 52.3 | 49.4 | (−) |
| Aged in deionized state | | 54.4 | 54.0 | 50.6 | (−) |

[a] All measurements were in 0.10 M KCl at pH *ca.* 5.5. [b] Reference values for 100% helix and 0% helix were −14,600 and −1900°, respectively (Tomimatsu *et al.*, 1966). [c] $b_0$ values for 100% helix and for 0% helix ($\lambda_0$ 218 mμ) were taken as −403 and +20°, respectively (Sogami and Foster, 1968). [d] $H_{225} = -A_{225+60}/19.9$; $H_{193} = A_{193-750}/36.5$ (Shechter and Blout, 1964).

Shechter and Blout (1964)) remains virtually the same on ageing. In contrast to this result, the aromatic Cotton effect near 290 mμ, which is best seen as a deviation from the linear Shechter–Blout plot in Figure 9, disappears almost completely on ageing. The Cotton effect presumably results from restrictions on the freedom of aromatic groups (Sogami and Foster, 1968). The population broadening resulting from ageing might thus be pictured crudely as representing some relaxation of the tertiary structure of members of the population without any significant alteration in secondary folding. This conclusion is in accord with our earlier observations that different subfractions of human and bovine plasma albumins do not differ significantly in helix content but that subfractions (at least in the case of the human protein) differ in the mean environment of the tyrosyl residues (Petersen and Foster, 1965c).

*General Remarks.* The above results suggest that disulfide interchange reactions are involved in the observed broadening of the population distribution of bovine plasma albumin. Admittedly, the evidence is circumstantial and more direct chemical evidence would be desirable. The fact that crystallized bovine plasma albumin is rendered more heterogeneous by storage at physiological pH and temperature is shown in Figure 10 and could mean that such a process takes place continuously *in vivo*. (On the other hand, it is possible that there exist in plasma SH blocking agents or other stabilizing factors which retard or prevent the effect.) This result also may imply that the protein as isolated had not attained fully the most random population distribution or, as seems equally possible, isolation and crystallization effected some artificial sharpening of the population. In any event it seems safe to conclude that permutations in the pairing of sulfur atoms through disulfide linkage are one, though not necessarily the only, contributing factor in the structural microheterogeneity of bovine plasma albumin.

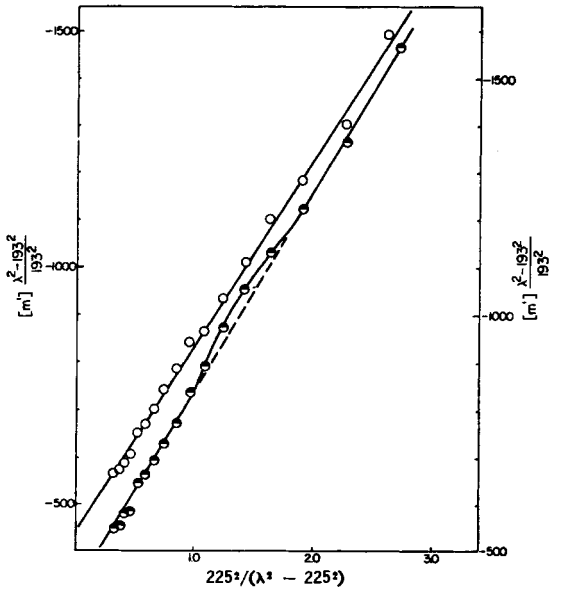


FIGURE 9: Shechter–Blout plots of optical rotatory dispersion data on charcoal-defatted bovine plasma albumin (half-filled circles and right-hand ordinate) and the same protein aged at pH 8.08, 2° for 86 hr without added salt (open circles and left-hand ordinate). The optical rotatory dispersion measurements were at isoionic pH (*ca.* 5.5) in the presence of 0.10 M KCl.

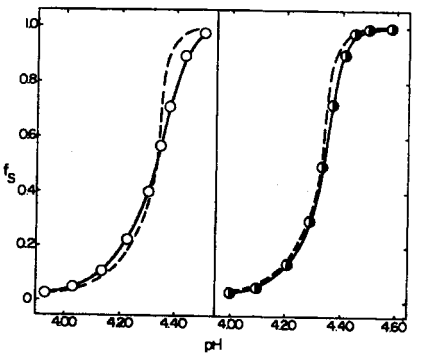


FIGURE 10: Solubility–pH profiles for bovine plasma albumin stored for 24 hr at pH 7.4 and a temperature of 38.5° to simulate physiological conditions. The left-hand panel shows profiles for bovine plasma albumin, the right-hand panel for iodoacetamide-blocked bovine plasma albumin. In both cases the dashed curves represent the controls.

The results summarized in Figure 4 demonstrate a sharp increase in the extent of the broadening reaction as the pH is raised from approximately 7.5 to 9.0. As suggested above, this might merely reflect ionization of the protein SH groups since SH-catalyzed disulfide interchange is generally thought to proceed *via* the ionized mercaptide form (Cecil and McPhee, 1959). However, there are reasons to believe that this is not the entire explanation. Katchalski *et al.* (1957) have examined the pH dependence of the reducibility of disulfide bonds in both human and bovine plasma albumins at 0°. They found no reduction to occur between pH 5 and 7. Between 7 and 10 approximately five disulfide bonds became available for reduction in the case of bovine plasma albumin and two in the case of human protein. The pH dependence of the exposure of these groups to reduction is remarkably similar to the pH dependence exhibited by Figure 4. Katchalski and coworkers concluded that a reversible pH-dependent transition takes place over this pH range. Moreover, Andersson (1966) observed that although approximately one disulfide bond was reducible in bovine plasma albumin at pH 8.00 in absence of added salt (Katchalski *et al.*, 1957), only two-tenths such bond was reducible in presence of 0.10 M Tris buffer plus 0.20 M NaCl. It was also reported by Davidson and Hurd (1967) that reduction of disulfide bonds of bovine plasma albumin by reduced glutathione increases dramatically with increase in temperature between 35 and 55°. These results accord well with the striking ionic strength and temperature effects described above for population broadening. Leonard *et al.* (1963) have presented evidence for a conformational transition in bovine plasma albumin over the pH range 7.0–9.0 on the basis of optical rotatory dispersion investigations. Benson *et al.* (1964) found an increase in the number of hydrogen atoms in bovine plasma albumin which exchange rapidly with deuterium over the pH range 5–8.5 and concluded there must be a change in conformation or in the equilibrium distribution of conformational isomers over this range. All of the facts are in accord with the postulate that there is such a shift in conformations, higher pH and decreased ionic strength tending to favor conformations of the bovine plasma albumin molecule in which internal regions are exposed. Such open forms are more reactive with regard to disulfide-bond reduction and intramolecular disulfide interchange, as well as possessing a higher degree of exposure of the peptide backbone to solvent.

In terms of this model, stabilization of bovine plasma albumin toward the ageing effect by bound fatty acids is readily understandable. It was shown long ago that bound fatty acid anions exert a profound stabilizing effect on albumins (Boyer *et al.*, 1964). There is a great deal of evidence that bound anionic detergents, at relatively low mole ratios, stabilize the compact (native) conformation of bovine plasma albumin (Foster, 1960; Markus *et al.*, 1964; Lovrien and Linn, 1967). Of particular interest is the work of Lovrien and Linn (1967) who studied the catalysis by bovine plasma albumin (and the human protein as well) of the *cis* → *trans* isomerization of azo dyes and concluded that conformational motility of the protein is involved in the catalysis. They observed detergent to suppress the catalytic effect and attributed this to a reduction in conformational motility. It is entirely possible that bound fatty acids, even at levels of the order of 1 mol/mol, might exert somewhat the same effect as bound detergent anions in these experiments.

These results and deductions are of particular interest in light of the currently popular concept that the three-dimensional structure of proteins is fully dictated by the primary structure. As one corollary of this "thermodynamic" concept of protein structure it has been assumed that there should exist a single most stable configuration of the disulfide bonds in those proteins possessing this structural feature. A great deal of support for this hypothesis has been presented by Anfinsen and his coworkers (Anfinsen, 1962), especially in the case of ribonuclease. That protein appears to possess one arrangement of the disulfide bonds which is favored overwhelmingly in the Boltzmann distribution between the various possible states. The present results suggest that in bovine plasma albumin, a much more complex protein and one possessing a very large number of disulfide bonds, no unique arrangement of the sulfur atoms is dominant. Rather, it seems likely that there exist a number of such pairings which have nearly equal stability so that the protein actually exists, even under physiological conditions, as a population of molecules with differing arrangements of the disulfide bonds. In a general way, the results accord with those of Krivacic (1966) who found bovine plasma albumin to yield an extremely broad population of monomeric molecules after reduction of the –S–S– bonds and reoxidation. It is possible that the relative abundance of the various species may be governed by thermodynamic considerations. It is well known that the plasma albumins are versatile binders of a wide variety of substances including fatty acids. Such binding might contribute alloplastic effects (Klotz, 1966) which modify the thermodynamic stability of various disulfide configurations. Thus the present results do not necessarily argue against the thermodynamic concept but only against the assumption that there exists a uniquely favored configuration. It might be suggested that the dictum "primary structure dictates three-dimensional structure" be modified only slightly to "primary structure dictates three-dimensional structure to a degree consistent with physiological function." For some proteins, such as enzymes, detailed specification of a unique structure may be essential; in the case of proteins of less well-defined function such as the plasma albumins a unique specification might be unnecessary or even undesirable.

Finally, it should be pointed out that the conditions found for the randomization of the disulfide bonds in bovine plasma albumin would not normally be encountered in the handling of preparations following crystallization. It therefore must be presumed that, to the extent to which fluctuations in disulfide pairing are responsible for the microheterogeneity, these structural variants are capable of coexisting in the crystalline state. It seems probable that the structural fluctuations are internal and without significant effect on the outward shape of the protein molecules. Clearly a number of in-

teresting questions are posed as to the actual crystalline state of albumins.

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