The Microheterogeneity of Plasma Albumins. IV. Evidence from Reversible Denaturation That Three-Dimensional Folding Is Not Responsible for Microheterogeneity*

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ABSTRACT: It has been suggested that differences in three-dimensional folding of otherwise identical molecules might be at least partially responsible for the observed microheterogeneity of plasma albumins. This possibility has been investigated by subjecting preparations of bovine plasma albumin and subfractions of known population distribution to drastic denaturing conditions and determining the distribution after renaturation. Solubility–pH profiles in 3 M KCl were employed for assessing population distributions. Unless the sulfhydryl group of the protein is blocked, a dramatic increase in microheterogeneity results on denaturation and renaturation, presumably due to disulfide interchange. Blocking of the sulfhydryl group of the protein by treatment with iodoacetamide produces no change in the solubility–pH profile or in the sedimentation and optical rotatory dispersion behavior. Charcoal-defatted protein so blocked is able to survive denaturation in 6 M guanidine hydrochloride even at elevated temperature (50°) or at low pH (2.45) with no detectable alteration of the population distribution. Nondefatted protein or protein defatted by the less effective conventional acid treatment yields a shift in distribution which is attributed to release of residual impurities under the denaturing conditions. Subfractions, differing in their population distributions, prepared from highly purified, charcoal-defatted, and sulfhydryl-blocked protein, retain their identity upon reversible denaturation. The results indicate that differences in three-dimensional folding *per se* are not an important source of microheterogeneity, and that some features of covalent structure must be involved. They are consistent with lipid impurities and permutations in disulfide pairing as contributing factors.

Studies of the N–F transition of plasma albumin have led to the conclusion that the N and F forms are resolved electrophoretically in spite of the fact that the isomerization appears to be immeasurably fast upon alteration of pH. This seemingly paradoxical behavior of plasma albumin has been reconciled on the basis of a microheterogeneity model (Sogami and Foster, 1963). The central postulate of the model is that plasma albumin consists of a distribution of species which differ in their propensity for undergoing the N–F transition. Evidence suggesting the model and the way in which it is capable of accounting for various observations have been discussed in some detail (Foster *et al.*, 1965).

Substantial support for the concept of microheterogeneity has emerged from a subsequent series of investigations conducted in this laboratory. The low solubility of the F form and the high solubility of the N form in 3 M KCl have been exploited in separating the original population into subpopulations by lowering of the pH in stepwise fashion. Subfractions prepared in this manner have been shown to differ in the pH range over which they undergo the N–F transition (Petersen and Foster, 1965a). Such subfractions also exhibit (Petersen and Foster, 1965b) significant differences in other properties such as hydrogen ion titration behavior, ultraviolet difference spectra, and resistance toward heat denaturation. On the other hand, optical rotatory dispersion measurements revealed that the Moffitt parameters were invariant among subfractions, suggesting no detectable differences in helical content.

Independently, Štokrová and Šponár (1963) proposed the concept of microheterogeneity in order to explain puzzling observations on thermal denaturation of plasma albumin. They concluded that plasma albumin consists of a population of molecules which differ in the characteristic temperatures at which they undergo heat denaturation.

In spite of the large body of evidence which supports the microheterogeneity model, the structural basis for this molecular nonuniformity has remained obscure. Among various possible causes considered earlier (Foster *et al.*, 1965) one interesting possibility is that of diversity in intramolecular noncovalent interactions through variations in three-dimensional folding. The present paper has as its major objective a clarification of this question. While the hypothesis that the amino acid sequence of a protein dictates a unique three-dimensional folding has received widespread acceptance, the hypothesis has not been rigorously tested with many proteins. In this regard it was felt that reversible

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disruption of noncovalent forces in known albumin populations should provide another test of this hypothesis.

Experimental Procedure

Materials. Crystallized BPA[1] was obtained from Armour Pharmaceutical Co. Three different lots (A70011, A69908, and B70411) were employed during this investigation. Lots A69908 and B70411 were essentially free of dimer (~2%) as judged from velocity ultracentrifugation. However, a significant amount of iron was found to be present in lot A70011. This iron contaminant apparently catalyzed the formation of excessive dimer, as defatted preparations invariably contained between 15 and 20% dimer. In order to circumvent dimerization caused by the presence of iron, plasma albumin was passed through a mixed-bed ion-exchange column, described by Dintzis (1952), before acid treatment and again after the defatting procedure. During the process of deionization it was discovered that most of the iron was also retained on the column.

IA and DTNB were obtained from Aldrich Chemical Co. Inc., and were used without further purification. Amberlite IR 120 and IRA 400 ion-exchange resins (Mallinckrodt Chemical Works) were employed for the purpose of deionizing bovine plasma albumin. Deionized water with a specific resistance of 10^6 ohms was obtained by passage of distilled water through a Bantam demineralizing column. Potassium chloride solutions were prepared from Fisher reagent grade KCl and were passed through a sintered-glass filter in order to remove visible particles.

Guanidine hydrochloride (Eastman Kodak) was recrystallized from methanol in the following way. A saturated methanolic solution of guanidine hydrochloride was prepared by adding excess guanidine hydrochloride to a relatively small volume of methanol and then carefully adding sufficient methanol until all guanidine hydrochloride was dissolved. The saturated solution was maintained at 45° with a constant-temperature bath. After saturation at 45° had been effected the solution was filtered to remove insoluble impurities and then allowed to recrystallize overnight at −5°. This procedure was repeated until solutions became clear or until most of the ultraviolet-absorbing materials disappeared. The crystals were dried in a vacuum oven to remove methanol. Since the nature and extent of impurities varied appreciably with each supply of guanidine hydrochloride, the number of required recrystallizations varied accordingly. However, in most cases two recrystallizations were sufficient.

Defatted, Deionized BPA. Two methods of defatting BPA were employed. In the first procedure, a modification of the method proposed by Williams and Foster (1959), 10 g of BPA was dissolved in 100 ml of deionized water and the pH of the solution was lowered to 2.7 by addition of 0.5 N HCl. The solution was then allowed to stand in the cold room (~2–4°) for approximately 3 days, after which the turbid solution was centrifuged at 10,000 rpm for approximately 30 min. The protein was filtered through a Millipore filter and then passed through a mixed-bed deionizing column. The deionized protein was lyophilized and stored for future use.

In the second procedure, a slight variation (Sogami and Foster, 1968) of the method described by Chen (1967) was employed for defatting purposes. The method consisted of dissolving approximately 8 g of BPA in 200 ml of deionized water and adding 6 g of Darco K.B. charcoal (dry weight) to the solution. The charcoal suspension was profusely washed with deionized water and filtered before it was used for defatting. The pH was lowered to 2.75 by addition of 0.5 N HCl. The solution was then stirred at 2° for a period of 4 hr after which it was centrifuged at 12,000 rpm to remove charcoal. After centrifugation the protein was passed through a Millipore filter and the pH was brought to 5.5 by addition of 0.1 N KOH. The protein was then passed through a deionizing column when experiments warranted deionized protein. It was found undesirable to lyophilize isoionic charcoal-defatted BPA because a small change in the solubility behavior invariably resulted (Sogami and Foster, 1968). It has also been noted that isoionic charcoal-defatted protein is highly unstable in solution when stored for a long period (Sogami and Foster, 1968). Because of this observation isoionic protein generally was used immediately after preparation. However, it was shown by Sogami and Foster (1968) that isoionic charcoal-defatted BPA is quite stable in 0.05 N KCl solutions for periods as long as 20 days. Therefore, salt was added to all solutions whenever immediate use was not anticipated.

Dialysis Tubing. Visking cellophane casing was boiled in half-saturated sodium bicarbonate solutions for approximately 3 hr. The cellophane casing was rinsed and boiled for a second time in sodium bicarbonate. The casing was then profusely rinsed and stored at 2° in deionized water. A third boiling was occasionally required to remove all water-soluble impurities.

In several experiments dialysis tubing of variable size was employed without discrimination. However, because of ill-defined correlations of size of dialysis tubing to alteration of solubility behavior, later experiments were confined to the use of dialysis tubing having an inflated circumference of 4.6 cm. Sogami and Foster (1968) found some broadening of the solubility–pH profile of BPA when larger size dialysis tubing was employed. Since apparent ageing effects due to dialysis tubing having inflated circumferences of 6.2 and 8.2 cm were not consistently demonstrated, the real cause of the apparent ageing of protein solutions under these conditions has remained obscure. It should be noted that all experiments reported herein in which questionable dialysis tubing was employed were checked and were shown to be free of complications caused by dialysis tubing.

[1] Abbreviations that are not listed in *Biochemistry 5*, 1445 (1966), are: BPA, bovine plasma albumin; IA, iodoacetamide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid).

Preparation of BPA Monomer. Sephadex G-150 gel was allowed to swell for 72 hr in deionized water with repeated decanting of the supernatant layer until the supernatant layer became clear. During the last 12 hr the gel was equilibrated with 0.1 M NaCl which was used as the eluent in all preparations of monomer. A 2.5 × 100 cm Sephadex laboratory column equipped with an upward-flow adaptor was obtained from Pharmacia Fine Chemicals. The column was poured at 2°; eluent was first permitted to flow downward for approximately 4 hr. The upward flow adaptor was then inserted, the column bed was adjusted, and upward flow was begun. The pressure head while packing the column as well as during actual operation was maintained at approximately 20 cm.

After allowing 0.1 M NaCl to pass through the column overnight, 10 ml of a 10% solution of BPA was applied to the column with the use of a syringe fitted with Tygon tubing. The flow rate remained constant at 17 ml/hr throughout the course of the experiment. The column was connected to a Gilson fractionator Model V-10 equipped with an ultraviolet monitor which was employed for detecting effluent protein. In most cases this procedure was sufficiently accurate for preparative purposes. However, tubes were read manually on a Hitachi 139 spectrophotometer when quantitative results were desirable.

Preparation of Subfractions. Two closely related methods were employed in the preparation of subfractions. In early studies the method described by Petersen and Foster (1965a) was followed without variation. Briefly, the method consisted of lowering the pH of a solution of plasma albumin in 3 M KCl to a desired value in the N–F transition region and allowing the protein to precipitate for a few minutes. The precipitate was removed by centrifugation and the process was repeated until the desired number of subfractions had been obtained.

The second procedure, applied generally to charcoal-defatted plasma albumin, was essentially that of Sogami and Foster (1968). Isoionic protein was dissolved in 3 M KCl and the pH was lowered into the region of the N–F transition. At the first sign of precipitation the protein was centrifuged to remove the precipitate which constituted the first subfraction. The pH was again lowered to the desired value in order to obtain the next subfraction. After each pH adjustment the solution was allowed to shake or stir for 12 hr to ensure complete precipitation. The notation used to designate subfractions was the same one adopted by Petersen and Foster (1965a).

When subfractions were dissolved in deionized water, the pH of the resulting solution was found to be extremely low, varying from 2.8 to 3.8. Sedimentation results revealed that fractions prepared in this manner contained large amounts of dimer. It is known that maximum dimerization of BPA occurs within this pH range (Williams and Foster, 1960). These findings necessitated the use of buffers in order to prevent excessive dimerization. When buffers were employed the precipitated fractions were dissolved in 0.2 M acetate buffer at pH 5.5. Subfractions were generally dialyzed against 0.05 M KCl before being used for solubility studies, since large volumes of acid would be required for solubility studies of buffered solutions.

Preparation of SH-Blocked BPA. An aliquot taken from a 0.2% stock solution of IA was added to a 0.1 M phosphate buffer solution of pH 7.0 after which an appropriate amount of bovine plasma albumin was added to make a final solution containing 10 moles of IA/mole of SH. The solution was then placed in the cold room at 2° and allowed to stand for 24 hr after which excessive iodoacetamide was removed by exhaustive dialysis.

In experiments where phosphate buffers were not employed, IA was added to an isoionic solution of BPA and the pH was raised to 7.0 by dropwise addition of 0.1 N KOH. Caution was always exercised in adding IA before adding protein to minimize the possibility of disulfide exchange.

Solubility–pH Profiles. In early experiments solubility data were obtained from experiments done according to the procedure described by Petersen and Foster (1965a). This approach proved satisfactory in most cases, but it was recognized that there were several experimental difficulties inherent in this method. Most notable were errors caused by adsorption of protein to fritted disk sealing tubes and systematic errors caused by dilution effects. Furthermore, the time required to obtain sufficient data was of relatively long duration. The problem of kinetics of precipitation also caused some concern.

In a second procedure suggested by Sogami and Foster (1968) many of the limitations of the first method were overcome. In this procedure a stock solution of 200 ml of 0.1% BPA in 3 M KCl was prepared. The pH was lowered into the N–F transition region by dropwise addition of 0.1 N HCl. A 9-ml portion of the mixture was withdrawn and transferred to an appropriately labeled 50-ml erlenmeyer flask. The procedure was then repeated throughout the precipitation range and the solutions were placed on a mechanical shaker and allowed to shake for 4 hr at 24 ± 1°. At the end of the shaking period the pH of each aliquot was accurately determined following the procedure of Sogami and Foster (1968). The samples were transferred to Beckman polyallomer centrifuge tubes and centrifuged at 25,000 rpm for 20 min in the Beckman Model L preparative ultracentrifuge. Prior to centrifugation, the refrigeration unit was turned on for approximately 5 min. This is sufficient time to prevent the rotor from heating excessively during the actual centrifugation. The clear supernatant were transferred to test tubes and 1 drop of 0.1 N KOH was immediately added to each tube to prevent aggregation. The tubes were then read directly in the Hitachi Perkin-Elmer 139 spectrophotometer.

Optical Rotatory Dispersion. Optical rotatory dispersion measurements were performed on a Bendix-Ericcson Polarmatic Model 460C recording spectropolarimeter. All studies were conducted at 24° empolying a 0.50-cm fused silica cell. Most studies were done at pH 5.5 in 0.2 M acetate buffer at protein concentrations of approximately 0.1%. In experiments where these conditions were not used the pH and

ionic strength were always adjusted to that of a control sample.

Optical rotatory dispersion data were analyzed by two different methods. In the first method data were analyzed according to the classical Moffitt–Yang (1956) equation

$$[M'] = a_0\left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}\right) + b_0\left(\frac{\lambda_0^2}{\lambda^2 - \lambda_0^2}\right)^2$$

where λ is the wavelength of light, a_0, b_0, and λ_0 are dispersion constants, and $[M']$ is the reduced mean residue rotation which is given by

$$[M'] = \frac{3M_0}{100(n^2 + 2)}[a]_\lambda$$

The dispersion data were generally analyzed by the statistical procedure described by Sogami *et al.* (1963), devised to find the best Moffitt parameters.

Estimation of helix content in this manner was generally adequate but on several occasions inconsistent results were obtained. Therefore, all results were checked using the method proposed by Simmons *et al.* (1961) in which the magnitude of the trough at 233 mμ is used to estimate the helix content of proteins. More specifically, the constants suggested by Tomimatsu *et al.* (1966) were used for estimation of helix content, namely, $[M']_{233}$ values of $-14{,}600$ and $-1900°$ for 100 and 0% helix, respectively. While it was pointed out by these investigators that there are obvious limitations in measuring helix from the trough method, this procedure proved quite consistent in giving reproducible results.

Denaturation of BPA. Bovine plasma albumin was denatured with guanidinium salts under a variety of conditions. Most experiments involved the denaturation of 0.5–1% solutions of plasma albumin at room temperature in the presence of 6 M guanidine hydrochloride and 0.2 M acetate buffer maintained at pH 5.2. Elevated temperatures and low pH were employed with guanidine hydrochloride in some denaturations and in early experiments 4 M guanidine hydrochloride was employed. In a few less well-defined experiments denaturation was effected in the presence of 4 M guanidine thiocyanate. In experiments conducted at elevated temperatures, constant temperature was maintained with a Haake Model F circulating bath. No attempt was made to rigorously exclude oxygen from any of the denaturing media.

Most samples were subjected to denaturing conditions for a period of 4 hr. Complete removal of the denaturant was achieved by exhaustive and repeated dialysis against a 40-fold excess of deionized water at 2° with mechanical agitation. Dialysis was repeated against fresh portions of deionized water until all salts had been removed, as judged from conductivity measurements on the dialysate. In experiments done at low pH the solution was brought to pH 5.5 before dialyzing out the denaturant.

Protein Concentration Determination. Concentrations of BPA solutions were routinely determined using a Hitachi Perkin-Elmer 139 spectrophotometer. The absorbance was measured at 279 mμ and BPA concentrations were calculated from the absorbance values assuming $E_{1\,cm}^{1\%}$ 6.67 at 279 mμ. For sulfhydryl analysis and optical rotatory dispersion experiments, concentrations were determined with a Cary 14 or Cary 15 recording spectrophotometer.

Sedimentation. Sedimentation experiments were performed in a Spinco Model E analytical ultracentrifuge equipped with RTIC temperature control unit and phase-plate schlieren optical system. All velocity runs were conducted at 20° and 59,780 rpm employing cells containing Kel-F centerpieces. The schlieren patterns were recorded on Kodak metallographic plates and were read in a Nikon Shadowgraph Model 6 C microcomparator.

Results and Discussion

The basic question asked in this study is whether the microheterogeneity of plasma albumins can be accounted for in whole or in part by differences in three-dimensional folding of various molecules which are identical in all other respects. Phrased another way, the question is whether different molecules possessing identical covalent structures can fold in differing stable or pseudo-stable three-dimensional structures leading to subtly different physical-chemical properties. Operationally, we adopted the premise that if the answer to either of these questions is yes, it should be possible to modify the microheterogeneity of a given sample of albumin by completely destroying its three-dimensional structure and then permitting the molecules to refold. To be meaningful, the experimental conditions must obviously meet two stringent requirements, namely, (1) the denaturing conditions must destroy all of the native folded structure and (2) all conditions used, both for denaturation and renaturation, must be such as to produce no alterations in the covalent structure of the molecules. On the basis of previous studies (Petersen and Foster, 1965a) solubility–pH profiles measured in 3 M KCl were adopted as a convenient and sufficient criterion as to the identity or nonidentity of the population distributions of samples before and after denaturation.

Results with Acid-Defatted BPA. In early experiments acid-defatted BPA was subjected to 4 M guanidine hydrochloride and regenerated protein was obtained by exhaustive dialysis against deionized water. Comparison of sedimentation and optical rotatory properties of renatured BPA revealed no significant changes in these properties upon reversal. It was noted, however, that appreciable amounts of dimer and other aggregates were formed during denaturation. A study of the solubility behavior of regenerated BPA is depicted in Figure 1. It is readily seen that the breadth of the N–F transition range is significantly greater for regenerated BPA than for native protein, indicating that an increase in heterogeneity accompanies denaturation and renaturation under the conditions employed. The question immediately arose as to whether this increase in heterogeneity could be directly ascribed to changes in folding *per se* or whether other undetermined factors

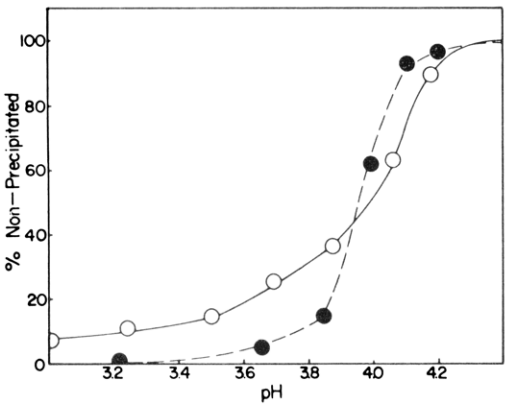

FIGURE 1: Solubility–pH profiles of BPA before and after denaturation in 4 M guanidine hydrochloride. (●) Acid-defatted control sample; (○) same protein denatured in 4 M guanidine hydrochloride and renatured. These data were obtained by the first procedure described in the text, namely, that of Petersen and Foster (1965a).

were contributing. It should also be noted in Figure 1 that there is a shift in the midpoint of the regenerated protein to higher pH. These findings warranted an assessment of various factors that might be manifested in the solubility behavior of BPA.

As shown by Petersen and Foster (1965a) the presence of dimer causes an alteration in the solubility behavior of BPA. Though the effect of dimer previously demonstrated was not as pronounced as the solubility change depicted in Figure 1, an assessment of the contribution of dimer was undertaken in this investigation. Some significant increase in dimer content invariably accompanied denaturation and renaturation under these conditions. Results obtained from a study of regenerated BPA in which dimer and other aggregates had been removed by exclusion chromatography indicated that this monomeric BPA exhibited the same general increase in heterogeneity as BPA containing dimer. While dimerization and polymerization may make some contribution to the broadening of the population distribution seen in Figure 1, it seems clear that some other factor predominates in rendering regenerated BPA more heterogeneous than the native protein.

Of even more concern than the dimer problem was the question of whether the observed broadening of the solubility–pH profiles might be attributed to sulfhydryl-catalyzed disulfide-exchange reactions. Petersen and Foster (1965c) demonstrated that broadening of the solubility–pH profile of BPA occurs on ageing solutions at pH above 7.0 and that this process can be inhibited by blocking the free sulfhydryl group of the protein. Krivacic (1966) demonstrated that reduction of the disulfide bonds in BPA followed by controlled reoxidation results in re-formation of considerable monomeric protein which, while possessing many of the properties of native BPA, is much more heterogeneous suggesting imperfect pairing in the resynthesis of the disulfide bonds. Several investigators (Huggins *et al.*, 1951; Kolthoff *et al.*, 1960) have demonstrated that disulfide interchange may occur in the denaturation of plasma albumins under some conditions. Since catalysis of the

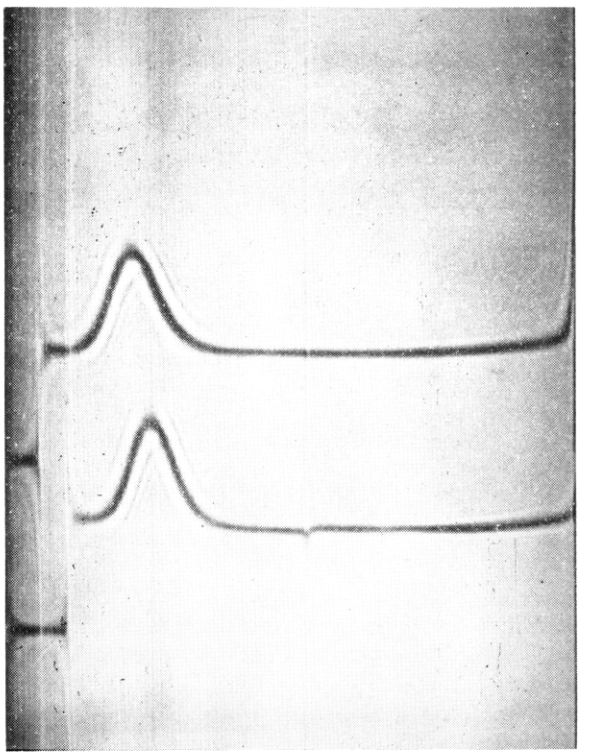

FIGURE 2: Sedimentation velocity patterns of IA-blocked BPA before and after denaturation. Upper pattern: protein regenerated after denaturation in 6 M guanidine hydrochloride; lower pattern: control sample of IA-blocked BPA prior to denaturation.

interchange is almost certainly effected by the ionized form of the sulfhydryl group (Cecil and McPhee, 1959), it might be inferred that the rate would be negligible at the pH employed in these experiments (5.2). Nevertheless, it was considered important to eliminate the possibility, in so far as possible, by employing BPA with the sulfhydryl group masked.

Experiments with Sulfhydryl-Blocked Acid-Defatted BPA. Efforts to circumvent sulfhydryl-catalyzed exchange reactions prompted modification of the sulfhydryl group by alkylation with iodoacetamide. The sulfhydryl titer of the modified protein was routinely assayed employing the procedure reported by Ellman (1959). Results indicated virtually complete disappearance of sulfhydryl groups upon alkylation. When the modified protein was characterized by optical rotatory dispersion and solubility behavior, no detectable changes in associated parameters were observed, indicating that the structural integrity of the native protein was unaltered after chemical modification. It is important to note that if side reactions do occur under alkylating conditions, such reactions are not manifested in the capacity for undergoing the N–F transition and can be neglected in this investigation. Actual solubility–pH profiles for sulfhydryl-blocked, acid-defatted protein are not shown. All the conclusions reached above will be substantiated in a later section devoted to experiments on charcoal-defatted protein.

Sulfhydryl-blocked acid-defatted BPA was denatured in 6 M guanidine hydrochloride. This higher con-

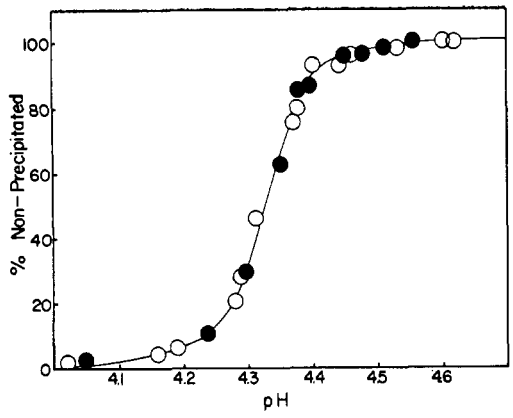

FIGURE 3: Solubility–pH profiles before and after blocking the sulfhydryl group. (○) Control sample of charcoal-defatted BPA; (●) same sample after blocking the sulfhydryl groups by treatment with IA as described in the text. These data and those in subsequent figures were obtained by the method of Sogami and Foster (1968).

centration of guanidine hydrochloride was chosen in view of the recent studies by Tanford and coworkers (Tanford *et al.*, 1966, 1967a,b; Nozaki and Tanford, 1967; Lapanje and Tanford, 1967), which have produced overwhelming evidence that the native structures of proteins in general and BPA in particular are completely destroyed in 6 M guanidine hydrochloride. Figure 2 shows a comparison of the sedimentation behavior of native SH-blocked BPA and the regenerated protein. The regenerated protein migrates as a single, relatively symmetrical boundary suggesting that all the dimer formed during earlier denaturation studies probably resulted from reactions occurring *via* the sulfhydryl group. More importantly, solubility studies (not shown) revealed virtually complete elimination of increased heterogeneity indicating that sulfhydryl-catalyzed disulfide-exchange reactions could account for at least most of the broadening of the solubility curves found in earlier experiments.

Experiments with Charcoal-Defatted BPA. In the early stages of this investigation we employed the defatting method developed by Williams and Foster (1959).

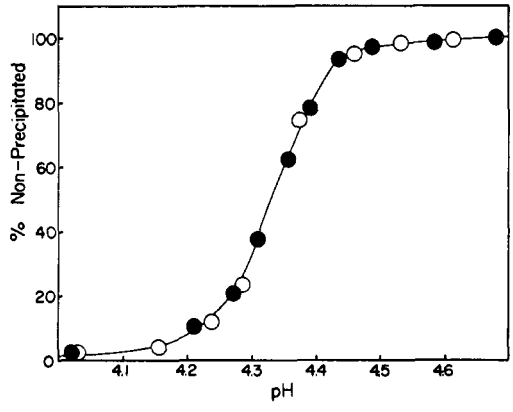

FIGURE 4: Solubility–pH profiles for IA-blocked charcoal-defatted BPA before and after denaturation. (○) IA-blocked charcoal-degatted BPA control; (●) same sample after denaturation in 6 M guanidine hydrochloride and renaturation.

TABLE I: Helix Content of Various Plasma Albumin Preparations as Estimated from $[M']_{233}$.

Protein Sample	$[M']_{233}$	Estd Helix Content (%)
Subfraction I (92–73)[a]	−8415	52
Regenerated fraction I	−8302	51
Subfraction III (24–1)[a]	−8150	50
Regenerated fraction III	−8280	51
Native BPA (undefatted)	−8443	52
Charcoal-defatted IA-blocked BPA	−8420	52
Charcoal-defatted IA-blocked BPA denatured in 6 M guanidine hydrochloride and renatured	−8290	51

[a] Numbers in parentheses give the limits, in terms of per cent nonprecipitated, over which the fraction was collected.

In spite of routine use of this method in our laboratory some concern persisted that all lipophilic contaminants might not be removed upon defatting and that partial or total loss of these impurities during denaturation and renaturation might contribute to the broadening of the population.

In a recent study Chen (1967) has shown that defatting effected by the adsorption of impurities to charcoal results in a more complete removal of residual contaminants. The increased efficacy of charcoal defatting was further demonstrated by Sogami and Foster (1968) who showed that charcoal-defatted BPA exhibits a sharper solubility curve than either undefatted BPA or BPA defatted by the earlier procedure. They also noted that charcoal defatting renders the protein very unstable on standing in salt-free solutions.

Figure 3 shows solubility–pH profiles for charcoal-defatted BPA and for the IA-blocked material obtained therefrom. Clearly modification of the sulfhydryl groups by the procedure employed here results in no detectable alteration of the apparent population distribution. Figure 4 compares the solubility–pH profiles of IA-blocked charcoal-defatted BPA before and after subjecting it to denaturation in 6 M guanidine hydrochloride and renaturation. It is striking to note that no perceptible increase in apparent microheterogeneity, and indeed no shift in the solubility–pH profile, accompanies this treatment. Further, though perhaps less dramatic, evidence for the complete recovery of the original native conformation is given in Table I which shows the optical rotatory dispersion parameter, $[m']_{233}$, to be the same within probable experimental error. These results strongly suggest that BPA does indeed assume a unique three-dimensional folding and that noncovalent forces are not a factor in microheterogeneity.

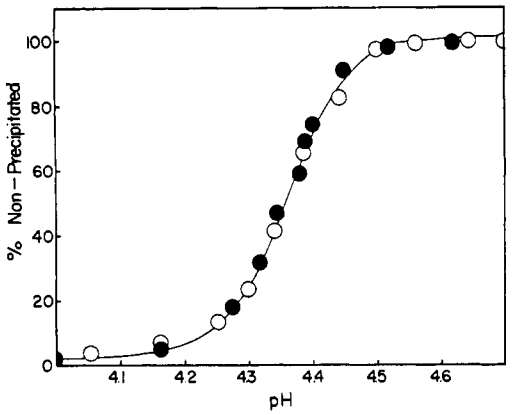

FIGURE 5: Solubility–pH profiles before and after denaturation at elevated temperature. (○) IA-blocked charcoal-defatted BPA control; (●) same sample after denaturation in 6 M guanidine hydrochloride at 50° and renaturation.

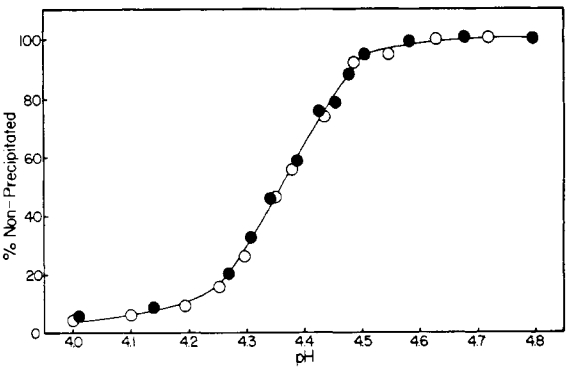

FIGURE 6: Solubility–pH profiles before and after denaturation at low pH. (○) IA-blocked charcoal-defatted BPA control; (●) same sample after denaturation in 6 M guanidine hydrochloride at pH 2.45 and renaturation.

There is, of course, no guarantee that all of the native folded structure has been destroyed under the denaturing conditions employed. Different molecules of the population could conceivably be folded in differing ways and retain sufficient "memory" of their initial state, even in 6 M guanidine hydrochloride, to guide them back to their initial state on renaturation. This seems unlikely in view of the previously mentioned experimental results of Tanford and associates (Tanford *et al.*, 1966, 1967a,b; Nozaki and Tanford, 1967; Lapanje and Tanford, 1967). Optical rotatory dispersion studies were carried out on charcoal-defatted IA-blocked BPA in the denaturing medium employed and essentially confirmed the published results of Tanford *et al.* (1967b). The b_0 parameter was found to be zero within experimental error; that is the dispersion obeyed the simple one-term Drude relation with $\lambda_0 = 221$ mμ.

Attempts were made to apply even more drastic denaturing conditions. Solutions of charcoal-defatted BPA that had been modified by reaction with iodoacetamide were maintained at either 37 or 50° for 2 hr in the presence of 6 M guanidine hydrochloride. The regenerated protein showed no change in solubility properties in either case. Results of the experiment at 50° are shown in Figure 5. The additional electrostatic stress imposed by low pH should certainly disrupt any residual structure that might have persisted in 6 M guanidine hydrochloride at pH 5. Sulfhydryl-blocked BPA was denatured at pH 2.45 in the presence of 6 M guanidine hydrochloride. The pH was brought to 5.5 after 2 hr and the guanidine hydrochloride was removed by dialysis. The results shown in Figure 6 again indicate that the same three-dimensional folding is recovered after denaturation under these extreme conditions.

These results strongly suggest that three-dimensional folding *per se* cannot be an important factor in the microheterogeneity of BPA. An alternative, though highly unlikely, interpretation of these results is that the distribution of molecules under consideration represents a most probable distribution. In this case, though reversible denaturation might alter the folding of individual molecules, the effect would be masked since no change in the over-all distribution would accompany reversible denaturation. It should be noted that the likelihood of obtaining a most probable distribution would depend upon isolating the protein without any fractionation during preparation. Thus, while the renatured protein might possess a most probable distribution such is probably not the case in the starting preparations, since in many cases crystallization yields are probably as low as 50%.

Solubility–pH Profiles of Native and Regenerated Subfractions. The ultimate test for a possible contribution of three-dimensional folding to the observed microheterogeneity is provided by an investigation of reversible denaturation of subfractions. Since subfractions exhibit characteristic distributions when subjected to denaturing conditions which disrupt noncovalent forces, they should lose their identity and assume or approach a most probable distribution, if differences in three-dimensional folding are indeed the basis for the observed microheterogeneity.

The population distribution of subfractions prepared by three slightly different procedures was studied before and after subjecting the subfractions to reversible denaturation. In all cases the sulfhydryl group was blocked with iodoacetamide.

In the first method subfractions were prepared from acid-defatted BPA and subsequently reacted with iodoacetamide. After removal of excess iodoacetamide by dialysis the subfractions were reversibly denatured by 6 M guanidine hydrochloride according to the procedure described previously. In the second procedure subfractions were prepared using charcoal-defatted BPA in which the equilibration period allowed for the precipitation of each subfraction was extended to several hours (see Experimental Section). In a third method subfractions were prepared from charcoal-defatted BPA in which the sulfhydryl group was blocked prior to subfractionation. The over-all result obtained from studies of reversible denaturation of SH-blocked subfractions was virtually the same with respect to all three methods of preparation. Subfractions prepared by the first method were most easily isolated but generally displayed broader pH–solubility profiles, probably

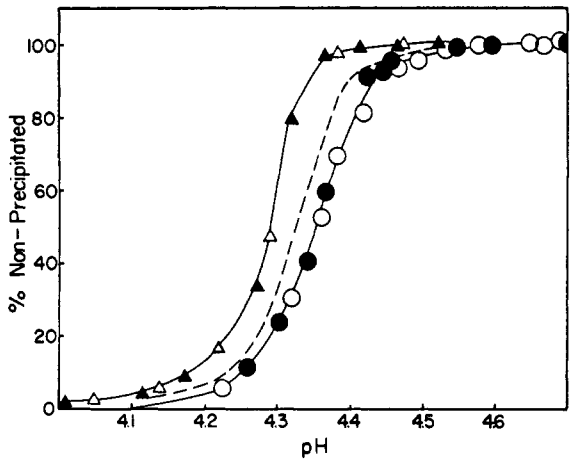

FIGURE 7: Solubility–pH profiles of BPA subfractions before and after denaturation. The dashed curve corresponds to the IA-blocked charcoal-defatted BPA employed as starting material. This albumin was subfractionated into two fractions of approximately equal weight after first discarding approximately the first 5% of the protein which precipitated at the upper range of the solubility–pH profile. Solubility–pH profiles for the two fractions were determined before and after denaturation in 6 M guanidine hydrochloride. Circles refer to the first subfraction, triangles to the second. Unfilled symbols refer to data obtained on the native fractions, filled symbols to data obtained on the denatured and renatured fractions.

because of a contribution of strongly bound lipid impurities.

Subfractions prepared according to the third method described above were invariably sharper with respect to the N–F transition than fractions prepared by other methods. Moreover these fractions exhibited markedly smaller differences with respect to their pH-dependent solubility behavior than did fractions prepared by the other methods. Nevertheless, reversible denaturation of these fractions probably provides the best proof that three-dimensional folding is not the cause of microheterogeneity. A representative comparison of optical rotatory properties of two extreme subfractions so prepared is given in Table I. It is seen that the helical contents are virtually identical in both cases, both before and after denaturation. This is in agreement with earlier studies from this laboratory which revealed that there was no detectable variation of helical contents among subfractions (Petersen and Foster, 1965b).

Figure 7 shows solubility–pH profiles obtained when charcoal-defatted BPA was subfractionated subsequent to blocking the sulfhydryl group. Shown also in Figure 7 are the results obtained from the reversible denaturation of these subfractions. It is seen that both fractions assume their original identity after denaturation and renaturation. Unfortunately, the difference between the two subfractions is somewhat exaggerated here because both profiles on fraction I were obtained at a protein concentration of 0.10% while those on fraction II were at 0.065%. Control experiments where protein concentration was varied indicated that the curve for fraction II should be shifted upward by 0.03 pH unit for strict comparison with the curve for fraction I.

These results strongly suggest that the microheterogeneity of BPA has a covalent origin. Doubtless bound contaminants make some additional contribution but this contribution must be very minor in preparations which have been defatted with charcoal. It should not be concluded that various molecules have exactly the same three-dimensional configuration even though they appear to have the same helix content within experimental error. But if they do in fact have differing three-dimensional structures these must be governed by differing covalent structural features which survive completely the drastic denaturation treatments employed. It must be emphasized again that this is so only if care is taken to preclude disulfide interchange by blocking the sulfhydryl groups. In a general way these results provide an additional demonstration, using a different and, we believe, very sensitive criterion, of the thesis that primary structure dictates a unique three-dimensional structure.

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Synthesis of [1-Isoleucine-, 3-Proline-, and 5-Alanine]-angiotensins. II*

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ABSTRACT: [1-Isoleucine,5-isoleucine]-angiotensin II and [3-proline,5-isoleucine]-angiotensin II were synthesized by the solid-phase method using dicyclohexylcarbodiimide as the condensing agent. The formation of the arginyl–proline bond was extremely difficult under conditions used. The former had about 25% pressor and 50% oxytocic activities giving further evidence the acidity of the β-carboxyl is unnecessary. The latter possessed 40% pressor and 80% oxytocic activities of the parent angiotensin. This relatively high biological activity was surprising because of the limitation on possible peptide conformations imposed by this cyclic amino acid.

[5-Alanine]-angiotensin II was prepared by solid phase using *N*-ethyl-5-phenylisoxazolium-3-sulfonate as the condensing agent. This peptide possessed approximately 5% of pressor activity of angiotensin II indicating the importance of branched side chain of valine or isoleucine occurring naturally in this position.

The β-carboxyl group of aspartic acid in angiotensin II is not essential for biological activity since its replacement by an amide group produced little change in biological activity (Rittel *et al.*, 1957; Schwyzer *et al.*, 1957). However, with β-aspartyl-angiotensin II, the side chain is lengthened by one methylene group and the duration of the pressor response is increased about 50% from that of the natural isomer (Brunner and Regoli, 1962; Regoli *et al.*, 1963). Since this apparent increase in pressor activity may be due to resistance to angiotensinase, it was of interest to determine the effect of a hydrophobic side chain at position 1 in angiotensin II. Position 3 of angiotensin II seems to have little side-chain specificity (Schwyzer *et al.*, 1957; Schwyzer, 1961; Khosla *et al.*, 1967a,b); therefore, proline was substituted here to modify the conformation of the peptide and determine the effect of this modification on biological activity.

Position 5 of angiotensin plays some role in biological activity, possibly because of binding of the aliphatic side chain on to the receptor protein as has been suggested for oxytocin (Rudinger and Krejci, 1962; Nesvadba *et al.*, 1963). Position of branching of side chain rather than its length appears to affect the activity. Thus, while 5-valine and 5-isoleucine, both with the branching at C_3, have similar activity, shifting the branching from C_3 to C_4 in leucine reduces the activity to 25% (Schwyzer and Turrian, 1960). Complete removal of all side-chain branching by replacing isoleucine with alanine in position 5 should further prove its importance.

Several workers have reported difficulties when using dicyclohexylcarbodiimide as a coupling reagent with amino acids containing an unprotected hydroxyl group (Bodanszky and Ondetti, 1966) or with amino acids such as asparagine and glutamine (Gish *et al.*, 1956; Ressler, 1956; Liberek, 1962; Paul and Kende, 1964; Kashelikar and Ressler, 1964). For these reasons we wanted to test the usefulness of Woodward's reagent K (*N*-ethyl-5-phenylisoxazolium-3-sulfonate) in solid-phase peptide synthesis using [5-alanine]-angiotensin II as the model peptide even though it contained neither of these amino acids.

Results and Discussion

The octapeptides [1,5-diisoleucine]-angiotensin II and [3-proline,5-isoleucine]-angiotensin II were synthesized by stepwise addition of succeeding amino acids to *t*-Boc-phenylalanine resin as described by Marshall and Merrifield (1965). The total amount of *t*-Boc-phenylalanine esterified to the polymer was determined by weight increase and by spectrophotometric estimation

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