The Microheterogeneity of Plasma Albumin. VI. Membrane Equilibrium Salting-Out As a Method of Demonstrating Microheterogeneity of Proteins*

Kin-Ping Wong† and Joseph F. Foster

ABSTRACT: A new method of demonstrating microheterogeneity of proteins has been developed based on the earlier salting-out studies of Mossé. Aliquots of protein are equilibrated across a semipermeable membrane against various concentrations of $(NH_4)_2SO_4$. The fraction of protein remaining in solution, f_s, is plotted versus the concentration of salt in the dialysate, Σ. Such solubility–Σ profiles are independent of the total protein concentration employed and are characteristic of the protein samples. This method is insensitive to lipophilic contaminants: nondefatted and defatted bovine plasma albumin samples have the same solubility–Σ profile. Subfractions have been prepared by stepwise membrane equilibrium precipitation with $(NH_4)_2SO_4$ and shown to have differing solubility–Σ profiles. That such subfractions are real and not artifactual is demonstrated by control experiments which show the solubility–Σ profiles to be entirely reversible and unaffected by prior precipitation of the protein. Such subfractions appear to be purely monomeric when prepared from monomeric bovine plasma albumin, and all have essentially the same SH content (*ca.* 0.60 mol/mol). Subfractions prepared by this method differ also in their solubility–pH profiles in 3 M KCl. Thus the microheterogeneity disclosed by the membrane equilibrium solubility method must be related to the microheterogeneity we have explored earlier and have attributed to differences in the stability of various members of the population with respect to the N–F transition.

The concept of plasma albumins as microheterogeneous populations of similar but nonidentical molecules was proposed by Sogami and Foster (1963) to account for the seeming paradox that N and F forms of the protein are resolved electrophoretically even though the interconversion of the two isomeric forms on changing the pH is very fast. Briefly, it was proposed that any given albumin preparation consists of a distribution of molecular species which differ in the pH at which they undergo the N–F transformation. Independently and almost simultaneously Štokrová and Šponár (1963) proposed that albumin consists of a distribution of species differing in their thermal denaturation temperatures. Further work from our laboratory has expanded on this idea (Foster *et al.*, 1965) and has demonstrated a relationship between the microheterogeneity based on the N–F transition and on propensity for thermal denaturation (Petersen and Foster, 1965b).

In an interesting thesis devoted to the phase relationships in protein–water–$(NH_4)_2SO_4$ systems, Mossé (1957) was led to the concept that horse serum albumin (as well as the other proteins he investigated, namely, pseudoglobulins and euglobulins) consists of a population of molecules differing in their solubility behavior. This result, unknown to us until recently,[1] is of considerable interest in view of the classical studies on protein solubility as a criterion of homogeneity and the fact that few if any proteins have ever passed this purity test (Cohn and Edsall, 1943). In essence, Mossé proposed that the dominant parameter in determining protein solubility is Σ, the concentration of salt in a protein-free solution in dialytic equilibrium with the protein solution. He suggested further that the usually observed logarithmic relation between protein solubility and salt concentration in fact represents the lower portion of a sigmoidal curve reflecting the heterogeneity of the protein with respect to the Σ for precipitation, and that the true solubility is a linear function of salt concentration.

The purpose of this study was to reexamine the salting-out behavior of BPA,[2] employing the best possible protein preparations, in the hope that a general method might be developed for assessing microheterogeneity in other proteins. The results substantiate the fundamental conclusions of Mossé and provide another method for subfractionating plasma albumin preparations and for comparing the microheterogeneity of such samples.

Experimental Section

Materials. Crystallized BPA was obtained from Armour Pharmaceutical Co. Lot No. B70411 was employed for most of the experiments. (Lots D71002 and D71209 were also used, but were not as thoroughly characterized.) This sample was essentially free of dimer or higher aggregates as judged by sedimentation velocity experiments. Polyacrylamide disc electrophoresis showed only a very faint dimer band. The

* From the Department of Chemistry, Purdue University, Lafayette, Indiana 47907. *Received June 11, 1969.* This work was supported by Grant CA-02248 of the National Institutes of Health, U. S. Public Health Service. Presented in part at the 165th National Meeting of the American Chemical Society, Atlantic City, 1968.

† Present Address: Department of Biochemistry, Duke University Medical Center, Durham, N. C. 27706.

[1] J. F. F. is indebted to Dr. Jacques Mossé for calling our attention to his interesting thesis and for helpful discussions of the results.

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

sulfhydryl titer was 0.65 as determined by the DTNB method (described in a later section). Fraction V BPA (Lot No. 46) was a product of Pentex Inc. and contained approximately 4% of α-globulin and 1% of β-globulin (Cohn *et al.*, 1946). The dimer content was 12–14% as judged by ultracentrifugation. Polyacrylamide disc electrophoresis showed that in addition to the characteristic monomer and dimer bands there were two or three very faint bands with slower electrophoretic mobilities.

Baker Analyzed reagent grade $(NH_4)_2SO_4$ was used. Concentrated solutions were clarified by filtering through fine grade sintered glass. Heavy metal ion contaminants were negligibly low. No significant ultraviolet absorption was detected. Vacuum-dried material was employed for calibration purposes. DTNB, obtained from Aldrich Chemical Co., Inc., was used without further purification.

Visking dialysis tubing was obtained from Union Carbide Corp. Size 20 tubing with a flat width of 0.984 in. and wall thickness of 0.0010 in. was used for all dialysis experiments. Amberlite IR 120 and IR A400 ion-exchange resins manufactured by Mallinckrodt Chemical Works were employed in deionization of protein. Deionized water with a specific conductivity of less than 10^{-6} mho was obtained by passage of distilled water through a Bantam demineralizing column (Model BD-1, Barnstead Still and Sterilizer Co.). Degassing was achieved by boiling.

Darko KB activated charcoal manufactured by Atlas Chemical Industries, Inc., was used. Water-soluble impurities were removed by washing profusely with deionized water. The dextran gels used, Sephadex G-150 and G-200, were products of Pharmacia Fine Chemicals, Inc. All other chemicals were the purest reagent grade products available.

Purification of BPA. Commercial samples of crystallized and fraction V BPA were defatted by the charcoal adsorption method of Chen (1967) as modified by Sogami and Foster (1968). Dimer and higher aggregates were removed by Sephadex G-150 exclusion chromatography following essentially the procedure described by Pedersen (1962). The protein was subsequently deionized by passing through a mixed-bed ion-exchange column (Dintzis, 1952) when isoionic protein was desired.

Membrane Equilibrium Salting-Out Studies. An exact amount (5 ml) of isoionic protein solution in 25% ammonium sulfate was measured into each of 12 dialysis bags and each bag was placed in a 50-ml screw-cap vial containing 25 ml of $(NH_4)_2SO_4$ solution. The concentration of salt ranged (in 0.5% increments) from 26 to 32%. Since different concentrations of $(NH_4)_2SO_4$ in water yield somewhat different pH values, all solutions were adjusted to the same pH (in the range 5.3–5.4) with dilute H_2SO_4 or NH_4OH. The vials were then sealed with Parafilm paper and stoppered with screw-caps. They were further protected from water leakage in the thermostat by placing in large rubber-stoppered test tubes. These tubes were clamped on a mechanism which rotated them end over end at approximately 5 rpm in a water bath at 25 ± 0.1°. It was found that a volume change occurred in the course of dialysis due to the initial difference of $(NH_4)_2SO_4$ concentration inside and outside of the dialysis bags. After dialysis the whole content of each of the dialysis bags was quantitatively transferred to a polyallomer centrifuge tube and the supernatant was separated from the precipitated phase by centrifugation in a Spinco Model L ultracentrifuge at 25,000 rpm for 25 min. Excess heating during ultracentrifugation was prevented by precooling the rotor chamber with the refrigeration system for 3 min. In this way the temperature was maintained at 25 ± 0.5° during centrifugation. The amount of protein remaining in solution was measured by quantitatively transferring the supernatant to a volumetric flask. The precipitate was resuspended in 8 ml of the $(NH_4)_2SO_4$ solution outside the dialysis bag, recentrifuged, and the washings were added to the supernatant. The resultant solution was diluted to a definite volume and its concentration was determined by spectrophotometry. Concentration of $(NH_4)_2SO_4$ outside the dialysis bag was determined by refractometry.

An accurate method for comparative membrane equilibrium salting-out profiles was used in later experiments. This method consisted of equilibrating two subfractions together with the control sample against the same $(NH_4)_2SO_4$ solution, thus eliminating discrepancies in pH, temperature, and other factors. In this case equilibration was carried out at room temperature (24 ± 0.5°) rather than in the water bath.

Membrane Equilibrium Subfractionation. Isoionic BPA was dissolved in an approximately 25% $(NH_4)_2SO_4$ solution and dialyzed for *ca.* 24 hr against an $(NH_4)_2SO_4$ solution of a Σ value desired for subfractionation. After dialysis the whole content of the dialysis bag was transferred to Beckman polyallomer centrifuge tubes and the supernatant fraction was removed by centrifugation in a Spinco Model L ultracentrifuge at 25,000 rpm for 25 min. The precipitated fraction designated "Subfraction I," was washed with 8 ml of the $(NH_4)_2SO_4$ solution from outside the dialysis bag to remove any occluded supernatant fraction. The concentration of the $(NH_4)_2SO_4$ solution outside the dialysis bag, Σ_I, was also determined accurately to locate the exact position of separation. Further subfractionation of the supernatant phase was achieved by repeating the above procedure at successively higher Σ values.

In this paper roman numerals are used to designate subfractions and in some cases subfractions are descriptively labeled by the precipitation range. Thus, a 1 to 0.9 subfraction means that the fractional amounts of the initial albumin remaining in solution at the beginning and end of the precipitation of the subfraction were 1 and 0.9, respectively. Another designation, describing subfractions in terms of the Σ range, would be more precise provided other variables in the subfractionation are carefully standardized. This designation is not used here since early subfractionation experiments were conducted at somewhat different pH and temperature values than later experiments.

Solubility–pH Studies. The method described by Sogami and Foster (1968) was adopted for all solubility–pH studies.

Determination of Reactive Sulfhydryl in BPA. The method was essentially that of Ellman (1959) employing DTNB. An approximately 0.1% solution of BPA in 0.1 M phosphate buffer (3 ml) was pipetted into the sample cell and the same amount of phosphate buffer into the reference cell. The absorbance at 279 mμ (for determination of the protein concentration) was measured employing the Cary 15 double-beam spectrophotometer. Equal volumes of DTNB (0.01 M in 0.1 M phosphate buffer) were then added to both cells and mixed well. The amount of DTNB used was slightly in excess of the stoichiometric amount. The absorbance at 412 mμ was followed with time using the same instrument with a Tungstun lamp. It was found that 20–30 min was sufficient for complete

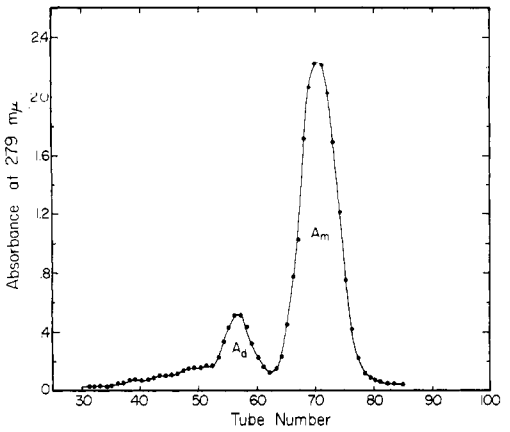

FIGURE 1: Sephadex G-150 exclusion chromatography pattern of BPA. The volume contained in each tube was approximately 4 ml. Components were separated on a 2.5 × 100 cm column using 0.1 M $(NH_4)_2SO_4$ as eluent.

reaction provided the pH of the system was never lower than 8.0. Care was exercised not to raise the pH high enough to cause hydrolysis of DTNB. The molecular weight for BPA in all these calculations was assumed to be 66,000 (Spahr and Edsall, 1964).

Sedimentation Velocity. All velocity runs were performed in a Spinco Model E ultracentrifuge at 20° and a rotor speed of 59,780 rpm. The instrument was equipped with phase-plate schlieren optical system and RTIC temperature regulator. Kel-F centerpieces were used in the cells. The schlieren patterns were recorded on Kodak metallographic plates and read in a Nikon Shadowgraph Model 6C microcomparator.

Determination of $(NH_4)_2SO_4$ Concentration. This was done by refractive index increment measurements, initially using the Brice-Pheonix differential refractometer. Approximately 1 ml of the sample solution was pipetted into the sample compartment of the partitioned cell with the reference cell filled with water. The temperature was maintained constant at 24 ± 0.1° by a circulating Haake thermostat connected to the jacket of the cell. This procedure was accurate and satisfactory except that the reading of refractive index increment was found to be tedious. In later experiments, a Bausch & Lomb Precision sugar refractometer was used to determine the concentration of $(NH_4)_2SO_4$ solutions. It was sufficiently accurate (±0.05% in salt concentration) and much less time was required.

Determination of BPA Concentration. Protein concentrations were routinely determined using the Hitachi Perkin-Elmer 139 UV-VIS spectrophotometer. The absorbance was measured at 279 mμ and BPA concentrations were calculated assuming $E^{1\%}_{1\,cm}$ 6.67 at 279 mμ based on dry weight and Kjeldahl nitrogen analysis previously performed in this laboratory.

pH Measurements. All pH measurements were made at room temperature with a Radiometer Model 25 pH meter equipped with a scale expander. A Beckman general-purpose glass electrode (41263) and a frit junction calomel reference electrode were used for most of the experiments. Occasionally a Radiometer combined electrode (type GK 2026C) was used and found to be satisfactory. The pH meter was routinely standardized against Sargent pH 4.01 and 7.00 buffers. Calibrations were also made with a pH 3.58 ± 0.01 buffer in 3 M KCl for pH determination in high salt media. This buffer was prepared by making a Sargent pH 4.01 buffer 3 M in KCl and then measuring its pH with a Radiometer type K-100 open liquid junction electrode. Thus errors caused by junction potential arising from high salt concentration were minimized.

Polyacrylamide Disc Electrophoresis. The method described by Ornstein (1964) and Davis (1964) was followed with minor modifications (Broome, 1963). The apparatus was constructed in this laboratory and reagents were supplied by Canal Industrial Co.

Treatment of Dialysis Tubing. Visking dialysis tubing was pretreated by washing with deionized water several times to remove glycerine and other water-soluble contaminants. The tubing was further boiled twice in approximately 1 M solution of $NaHCO_3$ for 1 hr each time, washed thoroughly with deionized water, and stored in deionized water in a cold room. Care was taken to avoid contamination of the tubing by the hands during experimental manipulation.

Results

Purification of BPA. Attempts were made to employ the most pure albumin sample attainable in these studies of microheterogeneity. The word "pure" as used here not only means the lack of contaminants in the protein sample, but also includes the absence of dimer or higher aggregates. Both fraction V albumin and crystallized albumin were employed as starting materials. The only difference between the two preparations is that the latter had been further purified from the former by crystallization with the aid of decanol or other long-chain alcohols (Cohn *et al.*, 1946). In addition further purification was conducted consisting of defatting, Sephadex gel exclusion chromatography, and deionization.

Defatting. It is a well-known fact that plasma albumin preparations invariably contain a significant amount of free (that is, unesterified) fatty acids bound to the protein with such avidity as to effect many physiocochemical changes. Attempts to remove these contaminants have been many. In addition, appreciable amounts of decanol or other long-chain alcohols have been observed by us to remain in the protein solution and are still there even after acid defatting. Lyophilization probably removes most of these contaminants. However, this procedure should be avoided as there is a possibility that irreversible change of the protein occurs on lyophilization (Sogami and Foster, 1968). In the experiments described here, almost all of the BPA samples were defatted by the modified charcoal adsorption method described in the Experimental Section. This treatment removes essentially all bound fatty acids and the nativeness of the treated protein is evidently retained (Chen, 1967; Sogami and Foster, 1968).

Preparation of BPA Monomer. The dimer and higher aggregates usually present in albumin samples have been shown to exert a profound effect on the solubility–pH profile (Petersen and Foster, 1965a; McMenamy and Lee, 1967). Thus the dimer biases the broadness of the albumin population as revealed by the solubility–pH profile and its presence might cast doubt on the validity of a genuine subfractionation. A typical separation of BPA by exclusion chromatography is shown in Figure 1. Sephadex G-150 was used exclusively

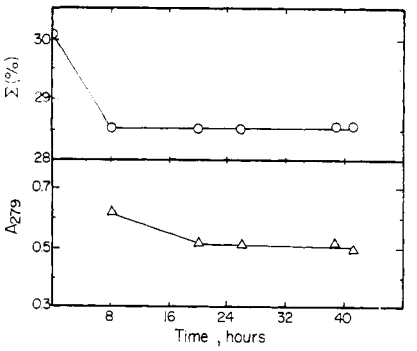

FIGURE 2: Time dependence of membrane equilibrium salting-out of BPA. Upper panel, the change of Σ with time; lower panel, the dependence upon time of the concentration of BPA remaining in solution, measured by its absorbance at 279 mμ.

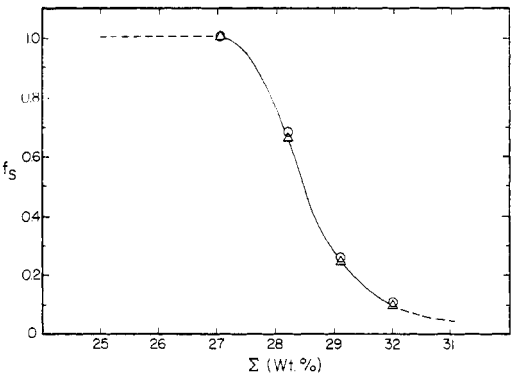

FIGURE 3: Precipitation and dissolution solubility–Σ profiles of BPA. Circles, precipitation; triangles, dissolution.

based on the fact that the monomer peak is better resolved from the dimer and higher aggregates than with G-200. In most isolations, a larger amount of protein was processed and separation was less distinct. But using the elution pattern in Figure 1 as a guide, pooled monomer fraction always exhibited a unqiue monomer band in polyacrylamide electrophoresis, and a single symmetric peak in the ultracentrifuge.

The exclusion chromatography of fraction V albumin showed an additional interesting feature. This protein sample usually contains traces of α- and β-globulins (Cohn *et al.*, 1946) which are probably responsible for the additional two or three faint bands in disc electrophoresis. These protein contaminants were removed during the isolation of monomer by Sephadex gel exclusion chromatography. Apart from this, some low molecular weight contaminants were retarded by the gel and removed concomitantly. It was observed that concentrated albumin solution after Sephadex treatment is less yellow in color and does not have the characteristic odor of decanol. In this regard, it should be noted that Sephadex, in general, has a tendency to adsorb aromatic compounds (Determann and Walter, 1968).

Deionization. Isoionic albumin was obtained by passing the protein through the mixed-bed ion-exchange column described by Dintzis (1952). Since no significant effect on solubility–Σ profiles was detected due to deionization, this procedure was not applied to many preparations. Moreover, loosely bound ions should be removed during the dialysis against approximately 25% $(NH_4)_2SO_4$ solution which preceded all solubility experiments.

The Membrane Equilibrium Salting-Out Method. All the studies described herein involve the ternary system protein–salt–water. Possible complications arising from the buffer component were thereby avoided. However, the pH values of all systems were rigidly controlled and checked. Bovine plasma albumin was employed throughout this investigation. Only $(NH_4)_2SO_4$ was used as the salting-out agent, though other neutral salts are also applicable. The reason for this choice has been twofold. On the one hand, the salting-out effect of sulfate is, in general, far greater than that of the corresponding chlorides. Full advantage can be taken of this high salting-out effect, because of the very great solubility of ammonium sulfate in water, which permits the attainment of extremely high ionic strength. Thus a much greater range of conditions for salting-out is attainable than with any of the other salts that have been employed. However, certain drawbacks inherent in its use should be mentioned. Ammonium sulfate is not a good buffer in the pH range below 8. Furthermore, the pH measurement of concentrated $(NH_4)_2SO_4$ solutions is complicated by a large junction potential.

All membrane equilibrium salting-out results were presented by plotting the fraction of protein remaining in solution, f_s, *vs.* the concentration of $(NH_4)_2SO_4$ in equilibrium across a semipermeable membrane. This concentration of ammonium sulfate is designated as Σ, and has its unit in weight per cent.

Time Dependence and Attainment of Equilibrium. This was studied by following the absorbance of supernatant at 279 mμ (A_{279}) and Σ in a representative system close to the midpoint of the solubility–Σ profile. These results are shown in Figure 2. On the basis of these and supplementary studies on other variables of the equilibrium, 20–24-hr equilibration was chosen for routine determination of solubility–Σ profiles. The attainment of equilibrium is further revealed by the fact that solubility–Σ profiles determined after 24 and 48 hr were identical.

Composition of the Solid Phase and Reversibility of Precipitation. It is likely that the solid phase was entirely amorphous in these experiments. In the first place, it is well known that BPA does not crystallize well from concentrated aqueous salt solutions. The salting-out of protein increases smoothly in a sigmoidal fashion on increasing salt concentrations, with no indication of the leveling-off which would be expected upon saturation with respect to a new solid phase. No crystalline form was detected on salting-out precipitation under the polarizing microscope. It is also noteworthy that the formation of a solid solution is rare in salting-out precipitation (Dixon and Webb, 1961).

In salting-out studies of serum proteins, Derrien (1952) reported that the order in which the ingredients are added to one another produces no difference in salting-out curves. In fact, he has presented experimental evidence that the salting-out precipitation curve and the dissolution curve are identical. However, in the same paper, he cautioned that solubility curves obtained by salting-out an amorphous protein from its solution, and by dissolving its crystals do not coincide at all. He showed that the dissolution curve of crystalline horse serum albumin is displaced toward lower salt concentration relative to the salting-out curve of the amorphous form.

We have tested for reversibility in our solubility studies,

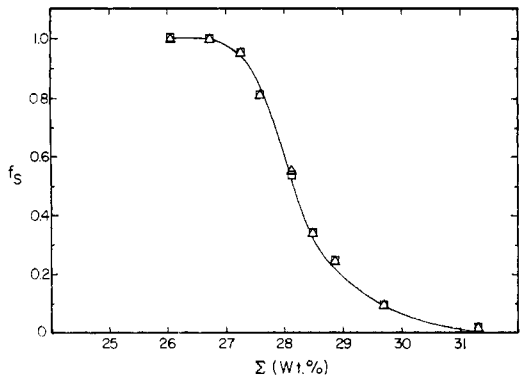

FIGURE 4: Comparative solubility–Σ profiles of charcoal-defatted BPA and the same protein after salting-out precipitation. Triangles, BPA; rectangles, BPA after salting-out and resolution.

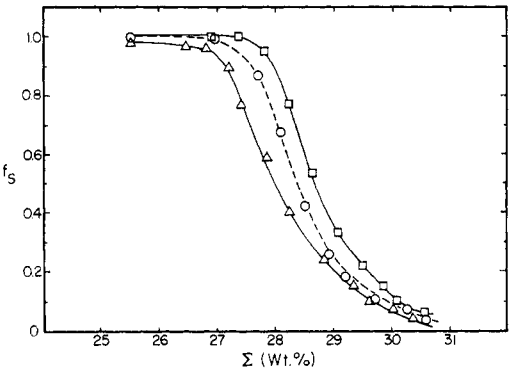

FIGURE 6: Solubility–Σ profiles for subfractions of untreated BPA. Circles, control BPA, triangles, least soluble subfraction; rectangles, most soluble subfraction. Each subsfraction constituted approximately one-third of the protein.

the results being shown in Figure 3. Both the precipitation curve and the dissolution curve were established simultaneously using the comparative solubility technique in the following manner. Equal amounts of protein from the same stock solution were placed in eight separate dialysis bags. The contents of four of the bags were completely precipitated by equilibrating with a 32% $(NH_4)_2SO_4$ solution. Then, each of these bags was matched with one whose content had not been precipitated, and each pair was equilibrated with a common reservoir of $(NH_4)_2SO_4$ solution. The Σ solutions were chosen to cover the whole range of the salting-out profile. The fact that the precipitation curve and the dissolution curve are identical clearly demonstrates the reversibility of the process.

Of more concern to us is the question: Can albumin which has been completely salted out and redissolved have the same solubility–Σ curve as that which has not been precipitated? This is especially crucial to the establishment of a true subfractionation procedure by membrane equilibrium salting-out. In this regard, we have obtained the comparative solubility profiles of a BPA sample which has been salted out from solution and one which has not. The identical character of the two curves shown in Figure 4 proves the reversibility of this method beyond any reasonable doubt.

Solubility As a Function of Total Protein Concentration. If the protein samples used were strictly homogeneous it is clear, from phase-rule considerations, that the concentration of protein dissolved at a particular salt concentration should be independent of the total concentration of protein added. That this is not so was clearly shown by Mossé and is amply borne out by our experiments. On the other hand, to the extent to which the hypothesis of Mossé is correct, the fraction of the protein remaining in solution should depend only upon Σ and not on total protein concentration.[3] The results shown in Figure 5 for both untreated and defatted BPA indicate that the solubility–Σ profiles are indeed independent of total protein concentration over a tenfold range. This result clearly demonstrates that neither protein sample is homogeneous and supports the concept that they are microheterogeneous. These concentration-independent sigmoidal salting-out curves can be rationalized as a result of a distribution of the microheterogeneous protein species in terms of the critical Σ value at which they precipitate. The results provide a basis for the subfractionation of the protein population and an unambiguous demonstration of microheterogeneity.

Additional interesting features are observed from comparison of the solubility–Σ profiles of untreated and defatted BPA (Figure 5). First, there is a very slight difference in the Σ midpoint of the two proteins. This small difference may be due to the small differences in pH at which the two curves were obtained (approximately 0.17 pH unit). The similarity of the curves for nontreated and defatted samples was unexpected. Previous N–F transition studies of plasma albumin in 0.1 M KCl by McMenamy (1965) indicated that the removal of bound fatty acids gave a higher order of pH dependence. This is further confirmed by the observation of Sogami and Foster (1967) and McMenamy and Lee (1967) that bound fatty acids contribute significantly to the microheterogeneity of BPA and that defatted BPA has a steeper solubility–pH profile and a midpoint which is shifted to higher pH value. In fact, the latter authors have attributed all observed microheterogeneity to the effect of bound fatty acids. It is conceivable that the effect of bound fatty acids is diminished at such high ionic strength as is used in the solubility–Σ method. In this regard, it is interesting to note that the solubility–pH

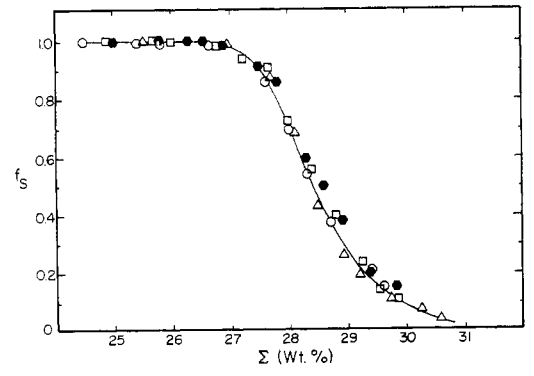

FIGURE 5: Solubility–Σ profiles of charcoal-defatted BPA as a function of total protein concentration. Rectangles, total protein concentration 0.46%; circles, 1.89%; triangles, 3.58%. For comparison the hexagons show results on 2% nontreated BPA.

[3] According to the results of Mossé, this simple relation breaks down at very low concentration. Our results indicate the approximation to be valid for BPA at total concentrations greater than *ca.* 0.5%.

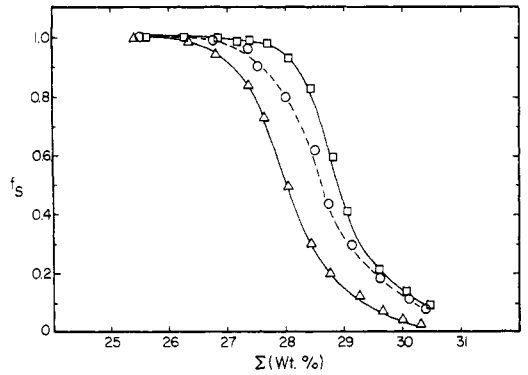

FIGURE 7: Solubility–Σ profiles for subfractions of charcoal-defatted BPA. Circles, control BPA; triangles, least soluble fraction; rectangles, most soluble subfraction. Each subfraction constituted approximately one-third of the protein.

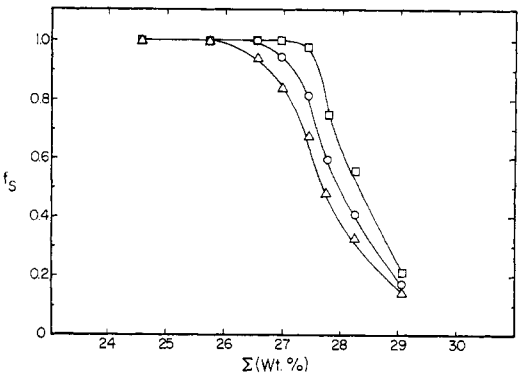

FIGURE 8: Comparative solubility–Σ profiles for subfractions of purified fraction V BPA. Circles, control BPA; triangles, least soluble subfraction; rectangles, most soluble subfraction. Each subfraction constituted approximately one-third of the protein.

profiles of BPA at 5 or 6 M NaCl are insensitive to the effect of bound fatty acids (M. Sogami, 1968, unpublished data).

Subfractionation and Comparative Solubility–Σ Studies of Subfractions. Mossé (1957) was able to fractionate the proteins he worked with into subfractions exhibiting differing salting-out properties. We have performed a large number of subfractionation experiments, following the procedure given under Experimental Section, and in every case have been able to prepare subfractions which have distinctly different solubility–Σ profiles. The results of three such experiments are shown in Figures 6, 7, and 8 which involve, respectively, non-treated crystallized BPA, charcoal-defatted monomeric crystallized BPA, and charcoal-defatted monomeric BPA prepared from fraction V without crystallization. In each case the fractionation was made into three approximately equal subfractions and the results shown are for the first (least soluble) and third (most soluble) of these, the intermediate one-third being discarded. Particular attention is called to Figure 8 which was conducted by the "comparative" solubility method, described under Experimental Section. In this case all three points at a particular Σ value were obtained by simultaneous dialysis against a common salt solution. In view of the earlier demonstration that precipitation *per se* is entirely reversible, that is does not modify the solubility–Σ profile, these results appear to provide a completely unambiguous proof of the microheterogeneity of the protein.

Correlation with Solubility–pH Profiles. We have so far demonstrated the microheterogeneity of BPA molecules beyond any reasonable doubt. Now the question can be raised as to the relation of this observed microheterogeneity to that demonstrated by the 3 M KCl solubility–pH method. The approach we adopted to answer this is as follows. BPA was subfractionated by the solubility–Σ method described earlier. Solubility–pH profiles of these Σ subfractions were then established. The rationale is that if these two methods are related, the solubility–pH profiles of Σ subfractions should differ in a systematic way. The results for charcoal-defatted BPA are shown in Figure 9, and suggest a relationship between the microheterogeneity detected by the two methods. Moreover, the Σ subfraction which was more resistant to salting-out was found to precipitate at a lower pH. It is also interesting that in this and all cases the curves merge at lower pH.

Heterogeneity and Microheterogeneity. We have considered the well-known heterogeneity of plasma albumin with respect to dimer content and free sulfhydryl groups. It will be seen that these two types of heterogeneity do not contribute to the observed microheterogeneity. Most BPA preparations contain a small amount of dimer. Its profound effect on the solubility–pH method has been mentioned (Petersen and Foster, 1965a; McMenamy and Lee, 1967). Commonly, dimer has a tendency to shift the midpoint of the solubility–pH profile to a higher pH value. It was also observed that when subfractionation was carried out by the solubility–pH method, dimers were generally enriched in early pH subfractions. We have studied the effect of dimer and higher aggregates on the solubility–Σ profile and the solubility–Σ subfractionation method. Subfractions prepared from monomeric BPA by the solubility–Σ method were assayed by polyacrylamide disc electrophoresis. No dimer was present. When BPA samples with a small amount of dimer were subfractionated by the solubility–Σ method, approximately the same amount of dimer appeared in all subfractions indicating that no disproportionation of dimer occurred during the process of subfractionation. Further evidence from ultracentrifugation studies of subfractions also supports this contention.

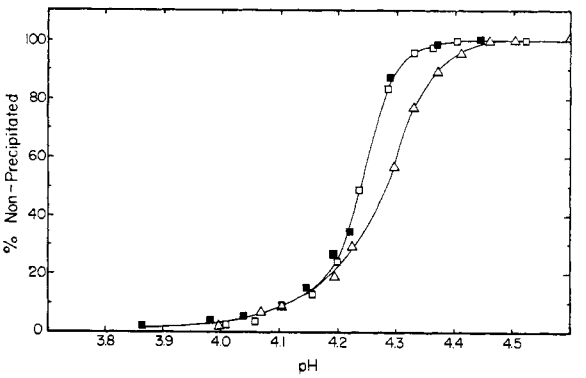

FIGURE 9: Solubility–pH profiles of Σ-subfractions of charcoal-defatted crystallized BPA. Triangles, least soluble subfraction; filled rectangles and unfilled rectangles, two separate experiments on the most soluble fraction. Each subfraction constituted approximately one-third of the sample.

Sedimentation velocity patterns of subfractions of monomeric BPA invariably showed a single symmetric peak indicating that no dimers are present in the subfractions.

Plasma albumin samples normally possess a "macroheterogeneity" in that they contain some molecules (typically 60–70%) which have one reactive sulfhydryl group while the remainder have this group masked, probably through mixed-disulfide formations (King, 1961). Foster *et al.* (1965) have concluded that this heterogeneity with respect to sulfhydryl is not responsible for the microheterogeneity observed in the solubility–pH profile. We have approached this problem by separating a BPA sample into five subfractions by the solubility–Σ method, and determining the sulfhydryl content of each subfraction using the DTNB method. These results are given in Table I. The constancy of sulfhydryl content among the first four subfractions clearly demonstrates that structural difference with respect to sulfhydryl is not a dominant factor in the observed microheterogeneity. The last (most soluble) subfraction does appear to have a significantly lower SH content than the bulk of the protein suggesting that a portion of the nonmercaptalbumin species possess a somewhat higher salt solubility than the average. Further evidence has come from the solubility–Σ studies of BPA with the sulfhydryl group blocked by iodoacetamide. The fact that this derivative has exactly the same solubility–Σ profile as nonblocked BPA reinforces the above conclusion that the state of the SH group is not a dominant factor. If the sulfhydryl group were involved in the population distribution manifested in the solubility–Σ profile, we would expect the two profiles to be different.

TABLE I: Typical Values of Sulfhydryl Content of Subfractions of BPA.

Protein Sample	Subfraction Range (Fraction Soluble)	Sulfhydryl Content
Control		0.593
Subfraction I	1.0–0.9	0.610
Subfraction II	0.9–0.74	0.636
Subfraction III	0.74–0.48	0.632
Subfraction IV	0.48–0.21	0.613
Subfraction V	0.21–0.09	0.548

It should be pointed out that the conclusion reached here does not apply to the more subtle and complicated involvement of the sulfhydryl group through intramolecular disulfide interchange under certain conditions. This has been investigated and will be presented in the next paper (Wong and Foster, 1969).

Discussion

Mossé (1957) demonstrated for horse serum albumin (as well as the other proteins he examined) that the amount of protein remaining in solution as a function of salt concentration is markedly dependent upon the total protein concentration employed. Thus no solubility curve can be established in the rigorous sense. He found, however, that for a given protein sample, precipitation would always be initiated when the salt concentration exceeded a concentration given by

$$S = \Sigma - P/a \qquad (1)$$

where S is the salt concentration, P the protein concentration, and a is a constant. This straight "threshold of precipitation" line he also termed the true solubility curve. His results indicated that this simple linear relation breaks down at low protein concentration (below about 1% with the protein he studied) but so long as consideration is restricted to the higher concentration range these true solubility lines have several remarkable properties. (1) The line of conjugation corresponding to the compositions of the two phases in equilibrium in the region of partial precipitation is identical with the true solubility line of the protein in the solution phase. On the other hand, the protein in the solid phase is completely precipitated anywhere along this line. Thus fractionation clearly accompanies partial precipitation. (2) Extrapolation of any such line of conjugation to the water–salt axis (protein free solution) yields Σ (eq 1) and this value is numerically equal to the salt concentration in a salt–water solution in dialytic equilibrium with the two-phase system. (3) For a given protein, if tie lines are generated by progressively increasing the salt concentration and increasing the relative proportion of precipitate, the slope ($-1/a$ in eq 1) changes systematically in such a way that all tie lines extrapolate to a common point on the protein–water axis (salt-free system). This point is taken to represent the hydration of the protein. Equation 1 then takes on a simple physical meaning, namely, that the total water in a given phase consists of two components, water of hydration which is substantially salt-free and water containing salt of composition Σ. It is the later parameter which determines the fractional degree of solubility of the sample.

The essential conclusions, from the point of view of the present paper, are that the protein is microheterogeneous and that Σ is a convenient parameter for characterizing the heterogeneity. The present results are in full accord with the results and interpretations of Mossé.

As noted above, Mossé found the simple linear equation (eq 1) to break down below protein concentrations of approximately 1%. Our results indicate that for BPA the law holds down to at least 0.5%. Thus Figure 5 shows that f_s, the fraction of the sample which is soluble, depends only upon Σ and is remarkably independent of the amount of protein added over an eightfold concentration range (from 0.46 to 3.58%). Some experiments done at approximately 0.2% did indicate a significant concentration effect, probably due to breakdown of the simple linear relation. Mossé (1957) suggested that in this low concentration range the familiar logarithmic solubility relation may take over.

Our results demonstrate the power of the Σ method for the study of microheterogeneity, at least in BPA. The solubility behavior is evidently a very sensitive function of some, as yet unknown, subtle variations in structural features of the various species. On the other hand, it is remarkable that the solubility–Σ profiles are relatively insensitive to other seemingly gross features. For example, the fatty acid contaminants normally present in BPA appear not to affect the profiles at all, as shown in Figure 5. There is apparently little difference in solubility behavior between mercaptalbumin and nonmercaptalbumin and blocking of the sulfhydryl group with IA

has no detectable effect. Most remarkably, we have found no evidence for any separation of monomer and dimer, though this question should be investigated further in samples containing more of the dimer component. While these results are puzzling, they only enhance the value of the technique for studying the unknown structural cause of microheterogeneity.

On the whole, the fractionation experiments were highly successful in that subfractions were obtained which have widely separated solubility–Σ profiles. On the other hand, the results are somewhat disappointing in that there is considerable overlap of the profiles for different fractions, especially at the high salt (low solubility) end. Very probably this results from occlusion or coprecipitation in the fractionation procedure. Mossé (1957) has argued that the dialysate solution is precisely the solution which should be used for washing the precipitate in such experiments since its composition, Σ, should be exactly that which dissolves the soluble fraction and does not dissolve the precipitated fraction. However, it must be recognized that in the washing step a situation probably arises in which the effective total protein concentration is too low for the simple linear law to hold. This effect would be in such a direction as to cause the solution of some protein which should have been insoluble at this Σ value. An effect in the opposite direction could result from coprecipitation, that is some interaction between soluble forms and insoluble forms which tends to hold the former in the solid phase. In view of these complications, it would be too much to expect that one could obtain nonoverlapping fractions from a single-stage fractionation of the type carried out here.

Finally, a word of speculation seems in order as to the mechanism of precipitation and the explanation for the existence of a critical solution behavior. To the extent to which the linear equation of Mossé is obeyed the implication is that at a given value of Σ some subspecies of the system are virtually infinitely soluble and the others virtually insoluble. Viewed from another point of view, a hypothetically homogeneous subset of molecules is virtually infinitely soluble below a certain Σ value and insoluble above. This is quite contrary to the usual solubility behavior of low molecular weight compounds. One possible explanation could be that there is a structural transconformation change involved in precipitation and that this transconformation change is a high-order function of the salt concentration. The fact that solubility profiles are correlated with low pH precipitability (the N–F transition) is suggestive of this possibility. Attempts were made to identify such a transition by making measurements of optical rotation at the 233-mμ trough as a function of salt concentration (Wong, 1969). Changes were seen in the range of precipitation but interpretation of such results is dangerous because of the possible influence of aggregation. Further studies of this possibility are in progress in our laboratory, as well as attempts to apply the solubility–Σ method to other proteins.

References

Broome, J. (1963), *Nature 199*, 179.

Chen, R. F. (1967), *J. Biol. Chem. 242*, 173.

Cohn, E. J., and Edsall, J. T. (1943), Proteins, Amino Acids and Peptides, New York, N. Y., Reinhold, Chapter 23.

Cohn, E. J., Strong, L. E., Hughes, W. L., Jr., Mulford, D. J., Ashworth, J. N., Melin, M., and Taylor, H. L. (1946), *J. Am. Chem. Soc. 68*, 459.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Derrien, Y. (1952), *Biochim. Biophys. Acta 8*, 631.

Determann, H., and Walter, I. (1968), *Nature 219*, 604.

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

Dixon, M., and Webb, E. C. (1961), *Advan. Protein Chem. 16*, 197.

Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.

Foster, J. F., Sogami, M., and Moore, W. E. (1967), *7th Intern. Congr. Biochem., Tokyo*, p 589.

Foster, J. F., Sogami, M., Petersen, H. A., and Leonard, W. J. (1965), *J. Biol. Chem. 240*, 2495.

King, T. P. (1961), *J. Biol. Chem. 236*, PC5.

McMenamy, R. H. (1965), *J. Biol. Chem. 240*, 4235.

McMenamy, R. H., and Lee, Y. (1967), *Arch. Biochem. Biophys. 122*, 635.

Mossé, J. (1957), *Ann. Inst. Natl. Rech. Agron., Ser. A*, No. 2949.

Ornstein, L. (1964), *Ann. N. Y. Acad. Sci. 121*, 321.

Pedersen, K. O. (1962), *Arch. Biochem. Biophys., Suppl. 1, 99*, 157.

Petersen, H. A., and Foster, J. F. (1965a), *J. Biol. Chem. 240*, 2503.

Petersen, H. A., and Foster, J. F. (1965b), *J. Biol. Chem. 240*, 3858.

Sogami, M., and Foster, J. F. (1963), *J. Biol. Chem. 238*, PC-2245.

Sogami, M., and Foster, J. F. (1967), *Federation Proc. 26*, 827.

Sogami, M., and Foster, J. F. (1968), *Biochemistry 7*, 2172.

Spahr, P. F., and Edsall, J. T. (1964), *J. Biol. Chem. 239*, 850.

Štokrová, Š., and Šponár, J. (1963), *Collection Czech. Chem. Commun. 28*, 659.

Wong, K. P. (1969), Ph.D. Thesis, Purdue University, Layfette, Ind.

Wong, K. P., and Foster, J. F. (1969), *Biochemistry 8*, 4104.