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Isomerization Reactions of Charcoal-Defatted Bovine Plasma Albumin. The N–F Transition and Acid Expansion*

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ABSTRACT: In view of the possibility that bound lipophilic impurities might make an important contribution to the observed microheterogeneity of bovine plasma albumin (BPA) samples, the character of the population distributions of albumin defatted by the conventional acid procedure and by the newer charcoal procedure has been determined by means of solubility–pH profiles and comparison was made with the nondefatted albumin used as precursor to these preparations. The results suggest that acid defatting leads to an artificial broadening of the population due to two factors, namely (1) partial but incomplete defatting and (2) some unknown irreversible alteration of some of the protein. The charcoal-defatted protein has a much narrower population distribution than either nondefatted or acid-defatted BPA. Improved procedures for determining solubility–pH profiles and for subfractionation of the populations were developed. The most striking property of the charcoal-defatted protein is its lability toward some reaction which results in broadening of the population. This process is most rapid above pH 7 but is appreciable even at the isoionic pH and at more acid pH in deionized solution, but is retarded by adding salt at concentrations as low as 0.03 M. Owing to the more homogeneous population, the acid transitions of charcoal-defatted protein are sharper and better resolved than in previous preparations. The N–F transition and acid expansion can be clearly resolved by optical rotation measurements at the trough of the first Cotton effect, 233 mμ, and by pH difference spectra. Careful measurements have been made of the optical rotatory dispersion properties of charcoal-defatted bovine plasma albumin both at neutral pH and through the range of the acid transitions. Hydrogen ion titration results are presented for the acid range which agree in general with previously published results but the number of titratable carboxyl groups (102) is in better agreement with amino acid composition than the number found earlier (108) for acid-defatted protein, suggesting the possibility that some deamidation may accompany the low pH treatment.

Formation of multiple boundaries in moving-boundary electrophoresis of plasma albumin near pH 4 was interpreted (Aoki and Foster, 1956, 1957a,b) as due to a pH-dependent isomerization reaction (N–F transformation). Resolution of the N and F forms in electrophoresis was explained by the present authors (Sogami and Foster, 1963) as due to microheterogeneity of crystallized plasma albumin. Specifically, we proposed that there exists, in any given albumin sample, a population distribution in terms of the characteristic pH at which various molecules undergo the N–F transformation. The concept of microheterogeneity of plasma albumins was proposed independently (Štokrová and Šponár, 1963) to explain results on thermal denaturation and was further supported by various experimental observations (Foster *et al.*, 1965; Petersen and Foster, 1965a,b).

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There are many possible sources of microheterogeneity such as bound impurities, variations in amide content, variations in amino acid composition or sequence, variations in disulfide pairing, and differences in secondary or tertiary structure (Foster *et al.*, 1965). Such structural variations might be present in the native protein or conceivably be introduced artificially during crystallization and purification. One of the important practical problems in the purification of crystalline serum albumin is the complete removal of impurities. Bound impurities, such as fatty acid and anionic detergents, have a strong effect on the conformation or conformational stability of plasma albumin (Ott, 1961; Markus *et al.*, 1964; Markus, 1965; Lovrien, 1963; Foster and Aoki, 1958). Furthermore, changes in the structure of plasma albumin molecules might be induced by defatting procedures. At least three methods for removing lipid impurities of plasma albumins have been employed, namely extraction by alkane–acetic acid mixtures (Goodman, 1957, 1958; McMenamy, 1965), acid treatment (Williams and Foster, 1959), and charcoal treatment in the acid pH region (Chen, 1967). It was reported by McMenamy (1965) that the hydrogen ion dependence of the transition of human plasma albumin from binding (with indole analogs) to nonbinding conformations was second order for nondefatted protein compared with approximately fifth order for albumin defatted by the alkane–acetic acid mixture. By considerations we have presented (Sogami and Foster, 1963; Foster *et al.*, 1965) this result implies the defatted protein to be more homogeneous than nondefatted protein. On the other hand, Chen (1967) concluded the charcoal-defatting method to have no effect on the pH dependence of acid-induced isomerization of either bovine or human albumins, according to tryptophan fluorescence experiments. In an attempt to resolve these paradoxical results we repeated the charcoal-defatting procedure of Chen and compared the properties of this protein with those of nondefatted albumin and albumin defatted by our standard procedure of acid treatment (Williams and Foster, 1959). The pH dependence of the N–F transition in 0.10 M KCl of charcoal-defatted BPA[1] was found to be steeper than that of BPA-defatted by the method of Williams and Foster (1959). Furthermore, charcoal-defatted BPA was found to be very unstable, in the deionized state, even in the neutral pH region. These observations prompted a careful study of several physical chemical properties of the protein with particular emphasis on the acid transitions.

Experimental Section

Preparation of Defatted Albumin. Crystallized BPA (lot B 70411) was obtained from the Armour Pharmaceutical Co. This sample of BPA contained approximately 5% dimer as judged by sedimentation velocity experiments in the Spinco Model E ultracentrifuge (protein concentration ~0.8%, 0.10 M KCl, isoionic). This BPA was defatted by two methods, that is the method of Williams and Foster (1959) and the charcoal treatment of Chen (1967). In the Williams–Foster method, BPA was defatted by lowering the pH of a solution to approximately 2.7 by adding diluted HCl and permitting the solution to stand at approximately 3° for 3–5 days. The lipid material could be removed as a separate phase by centrifugation or by filtration on a Millipore membrane (0.45 μ). The clear, acidic BPA solution was deionized by passing through a Dintzis column (Dintzis, 1952). Conventionally defatted deionized BPA was very stable for at least 20 days in the cold room. In general, the dimer content of conventionally defatted deionized BPA was slightly higher (8–10%) than that of nontreated BPA. However, when nontreated BPA was first deionized and then defatted by the Williams–Foster method, no significant increase in dimer content was observed. All of the conventionally defatted deionized BPA in this report was prepared by this modified method.

In the charcoal treatment, the authors slightly modified the defatting procedure of Chen (1967). Approximately 5 g of BPA was dissolved in 100 ml of deionized distilled water and then wet charcoal (Darco KB) was added to the solution (BPA–charcoal ratio of 4:3 dry weight). Prior to use, the charcoal was stirred in deionized distilled water, filtered using a Buchner funnel, and washed with deionized distilled water on the funnel. The BPA–charcoal mixture was stirred to obtain a homogeneous suspension, and the pH of the suspension was then adjusted to 2.75 by adding 0.5 M HCl slowly and dropwise. The acidified suspension was stirred (Teflon-coated magnetic stirrer) for 4 hr in a cold room using sufficient speed to suspend all of the charcoal without foam formation. Then, the mixture was centrifuged at 14,000 rpm for 30 min in the cold room, using a Sorvall Model SS-3 centrifuge. The clear supernatant was recentrifuged under the same conditions or was filtered using a Millipore membrane (0.45 μ). The pH of the BPA solution was adjusted to 5.4–5.5 by adding 0.10 M KOH, to provide protection against acid ageing. Recovery of BPA following the charcoal treatment was about 75%. After charcoal treatment, addition of salt to the BPA solution is extremely important to protect against alterations in the heterogeneity during storage in the cold room.[2] Charcoal-defatted BPA solutions should contain at least 0.03 M KCl or NaCl at pH 5.4–5.5 and 0.15 M KCl or NaCl at pH 7.5. Charcoal-defatted BPA was stable for 20 days in the cold room at pH 5.4 in the presence of 0.03–0.04 M KCl. Prior to use, charcoal-defatted

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: BPA, bovine plasma albumin; HSA, human serum albumin; HMA, human mercaptalbumin; HNA, human nonmercaptalbumin; SDS, sodium dodecyl sulfate; AD_m, albumin–detergent complex containing m equivalents of SDS ion per mole of protein; PMB, *p*-hydroxymercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); IA, iodoacetamide.

[2] Changes of conformation in the deionized state were mainly manifested in the solubility–pH profile. There was no change in secondary structure by optical rotatory dispersion analysis. Ageing phenomena during storage at isoionic and around neutral pH will be given in a future report. When the flow rate in the Dintzis' column is very slow, it might be advisable to add an exact known amount of KCl or NaCl to the flask which receives the effluent.

BPA was deionized on a Dintzis column. After deionization, the salt concentration was adjusted to the desired concentration as rapidly as possible.

Blocking and Determination of Sulfhydryl Groups. Blocking of SH groups was carried out by stirring slowly with 3 moles of iodoacetamide (Sigma Chemical Co.)/mole of protein at pH 7.00 in 0.035 M KCl at 2–3° for 24 hr. The solution was then dialyzed several times against 80 volumes of deionized water. However, it might be better to react with iodoacetamide in the presence of 0.20 M KCl or NaCl to protect against possible disulfide interchange during the course of the reaction.

Reactive SH groups were determined by titration with PMB (sodium salt, Sigma Chemical Co.) at pH 4.63 (Benesch and Benesch, 1962) or DTNB (Aldrich Chemical Co.) in pH 8.00, 0.10 Γ/2, phosphate buffer (Ellman, 1959) assuming a molecular weight of 66,000. However, in the later method the pH of the BPA solution dissolved in pH 8.00, 0.10 Γ/2 phosphate buffer was lower than 8.0, because of the low buffering capacity of the phosphate buffer. Therefore, by separate experiment, the amount of 0.10 M KOH required to bring the pH of a given BPA solution to pH 8.00 was exactly evaluated before starting the SH analysis.

Solubility–pH Profiles in 3.0 M KCl. Our previous filter-stick method (Petersen and Foster, 1965a,b) was not used for solubility–pH profiles because of the slowness of precipitation and errors due to adsorption of BPA on the sintered-glass filters. We devised a new shaking method as follows. The pH of 200 ml of 0.08–0.1% BPA solution in 3.0 M KCl was adjusted to various values by adding 0.10 M HCl or 0.10 M KOH (total chloride concentration adjusted to 3.00 M by KCl). The pH values were decreased stepwise from 4.7 to approximately 3.7 with 0.04–0.05-pH intervals. At each pH value an 8–9-ml aliquot was taken and delivered into a 50-ml erlenmeyer flask. The flasks were sealed with parafilm. These solutions were shaken for 3.5–6 hr at 25° at approximately 1 cps. After shaking, the pH value of each flask was measured with a Radiometer Model 25 pH meter with expanded scale. In general, the pH change on shaking was positive in the precipitating pH range and almost zero in the nonprecipitating pH ranges. After pH measurement, the turbid BPA solutions were centrifuged at 25,000 rpm for 20 min using a Spinco Model L preparative ultracentrifuge at 23–27°. For temperature adjustment of the rotor, the cooling system of the ultracentrifuge was operated for approximately 5 min, just before inserting the rotor. During running, the cooling system was not operated. The concentration of protein in the supernatant solution was determined by measuring the optical density at 279 mμ (assuming $E^{1\%}_{1cm}$ 6.67) after adding 1 or 2 drops of 0.10 M KOH to remove any turbidity. The pH–solubility profiles showed extremely high reproducibility (discrepancies between two independent experiments were less than 0.005 pH). The solubility profiles determined by the shaking method were less steep than those by the filter-stick method in the initial region of precipitation.

Subfractionation of BPA. The subfractionation method used earlier (Sogami and Foster, 1963; Petersen and Foster, 1965a,b) was not applicable for charcoal-defatted BPA, because of the extreme steepness of the solubility curve (W. E. Moore and J. F. Foster, unpublished observations). The following method, which permits adequate time for equilibration, has been found to yield distinct subfractions.

The solubility–pH profiles in 3.0 M KCl were evaluated at a protein concentration suitable for fractionation, namely 0.5% or less. Using this solubility–pH profile as a guide, pH values of aliquots were adjusted to the desired pH values depending on the subfractionation desired. After shaking for 5–6 hr at 25°, centrifugation was carried out at 15,000 rpm using a Sorvall Model SS 23 centrifuge in the cold room. The precipitate was dissolved in deionized water, keeping the pH value of the solution higher than 4.5 during dissolution. The pH was finally adjusted to 5.4–5.5 by adding 0.10 or 0.20 M KOH. The solution was dialyzed against 0.10 M KCl to protect against ageing effects. For determination of solubility–pH profiles of supernatant fractions the pH of the supernatant was readjusted to 5.4 by adding 0.10 M KOH (total chloride strength 3.0 M by KCl), and then the solubility–pH profile was determined using the shaking method described above.

pH Measurements. Measurements of pH were made with a Radiometer type 25 (expanded scale) pH meter using a Beckman general-purpose glass electrode (41263) and frit-junction reference electrode or using a Radiometer combined electrode GK 2026 C at 25°. The pH meter was standardized against Sargent pH 4.01 and 7.00 buffers. For determination of solubility–pH profiles it was necessary to calibrate reference electrodes frequently because the pH standards were only 0.05 M and pH measurements were in 3.0 M KCl. Serious errors were encountered with older reference electrodes and are attributed to junction potentials arising from fixed charges on the surface of the porous material of the frit junction due to adsorption of proteins or chemicals. The junction potential was tested using pH standards in 3.0 M KCl which were also measured using an open liquid junction reference electrode (Radiometer). In the authors experiments, both GK 2026 C and Beckman frit junction reference electrodes had to be replaced two to three times per year because of these difficulties. A pH–titration curve of charcoal-defatted BPA in 0.10 M KCl at 25° was determined using 30.0 ml of 0.576% charcoal-defatted deionized BPA, a microburet of 0.2500-ml capacity (L. S. Starrett Co.), and jacketed beaker. Titration data were analyzed assuming the molecular weight of BPA as 66,000. Electrostatic correction for the estimation of pK_{int} was carried out using mobility data for conventionally defatted BPA in 0.10 M chloride (Vijai and Foster, 1967).

Optical Rotation. Optical rotatory dispersion and optical rotation at 313 and 233 mμ were carried out using a Polarmatic Model 460C spectropolarimeter (Bendix Ericsson). The spectropolarimeter was adjusted to overcome false Cotton effects (Resnik and Yamaoka, 1965) by the Bendix Co. Wavelength calibration of the instrument was made using a relatively slow scan speed of 500 $\bar{\nu}$/min, chart speed 1 in./min, and slit width 0.20 mm using a mercury arc lamp. Calibration of optical rotation was carried out using sucrose (Mallinckrodt ana-

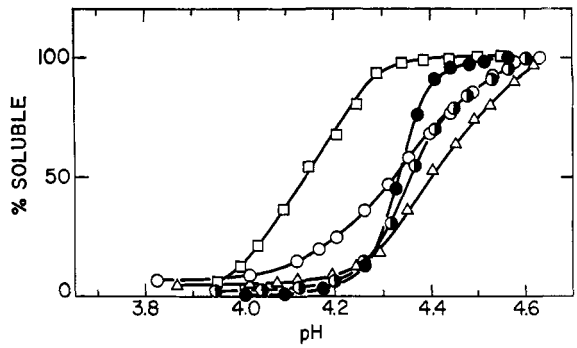

FIGURE 1: Solubility–pH profiles in 3.0 M KCl. (□) Non treated BPA; (●) charcoal-defatted BPA; (○) conventionally defatted BPA; (◑) charcoal-defatted BPA subsequently acid treated for 4 days at pH 2.245 at 2–4°; (△) conventionally defatted deionized BPA subsequently redefatted by charcoal.

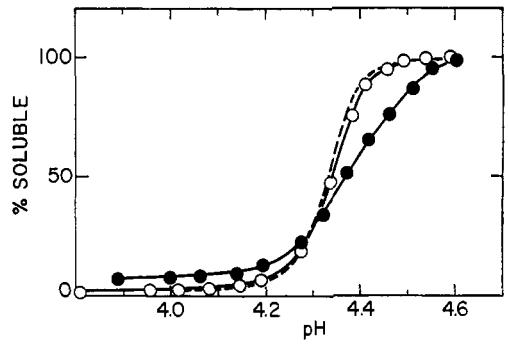

FIGURE 2: Solubility–pH profiles in 3.0 M KCl. (- - -) Charcoal-defatted (deionized) BPA; (○) iodoacetamide-treated (pH 7.00) charcoal-defatted BPA; (●) charcoal-defatted deionized BPA aged for 2 weeks in the deionized state at 2–4°.

lytical reagent) at 18.3 and 25 × 10^3 $\bar{\nu}$ (slit width: entrance, 0.50 mm; exit, 0.40 mm; scan speed 500 $\bar{\nu}$/min). Optical rotatory dispersion was carried out from 22 to 39 × 10^3 $\bar{\nu}$ using scan speed 2000 $\bar{\nu}$/min, slit width 0.5 mm (entrance), 0.4 mm (exit), and compressed mode 50 or 100. Optical rotation readings at fixed wavelength, such as 313 or 233 mμ were carried out using slit width 0.5 mm (entrance), 0.4 mm (exit) at 313 mμ and 1.0 mm (entrance), 0.80 mm (exit) at 233 mμ, scan speed 500 $\bar{\nu}$/min, and compressed mode 50. Cells for optical rotation or for optical rotatory dispersion were 0.504-, 2.002,- and 3.000-cm jacketed cells. All rotation measurements were carried out at 24 ± 1°. Care was taken that the indicated transmittance was always greater than 40%. Optical rotatory dispersion data were analyzed using the Moffitt–Yang equation (Moffitt and Yang, 1956) and the Schechter–Blout equation (Schechter and Blout, 1964a,b; Schechter *et al.*, 1964) assuming a mean residue weight of 118 g $mole^{-1}$. The refractive index correction for a given wavelength in millimicrons was calculated by the following relation for pure water at 20° (Dorsey, 1940) (though real experiments were at 24°), $n^2 = (1.762530 - 1.33998) \times 10^{-8} \lambda^2 + (6309.57/(\lambda^2 - 15880.0))$.

Miscellaneous. Difference spectra were determined by scanning from 350 to 260 mμ using a Cary 14 M spectrophotometer (Applied Physics Corp.), protein concentration 0.27% in 0.10 M KCl, ambient temperature 24°. Protein concentrations were determined by absorbance in a Hitachi Perkin-Elmer 139 spectrophotometer. The extinction of BPA was assumed to be $E_{1\,cm}^{1\%}$ 6.67 at 279 mμ. The molecular weight of BPA was assumed as 66,000 for titration and SH analysis. The dimer and polymer contents of BPA in 0.10 M KCl were analyzed by sedimentation velocity patterns at 20°, 59,780 rpm in a Spinco Model E ultracentrifuge. All deionized water employed was prepared by passing distilled water through a mixed-bed column (Barnstead Bantam Model BD-1) and was found to have a specific resistance greater than 10^6 ohms. Conductivity of charcoal-defatted deionized BPA was measured using a Radiometer Model CMD conductivity meter. Cellophane tubing (Visking) was pretreated by boiling in 50% saturated $NaHCO_3$ (half-saturation at 25°) and by sufficient washing with deionized distilled water. Pretreated cellophane tubing was stored in deionized water in a cold room to suppress growth of bacteria.

The HNA employed in one experiment was derived from mercury-free HMA, prepared in this laboratory by the method of Dintzis (1952), by blocking of the sulfhydryl group by exchange with cystine as described by Andersson (1966).

Results and Discussion

Solubility–pH Profiles. Solubility–pH profiles (in 3.0 M KCl) of charcoal-defatted and conventionally defatted BPA are shown in Figure 1. The midpoints of the profiles for the two defatted samples are virtually the same, namely 4.330 ± 0.005 for the charcoal-defatted and 4.325 ± 0.005 for the conventionally defatted samples, both far above the value for the nontreated BPA used as starting material. The relative degree of microheterogeneity, as judged by the breadth of the profiles, is greatest for the conventionally defatted and least for the charcoal-defatted protein. In more quantitative terms, ΔpH_{10}^{90} (Petersen and Foster, 1965a,b) is 0.30 for the nontreated, 0.48 for the conventionally defatted, and only 0.17 for the charcoal-defatted samples.

As will be seen in the next section, the difference in the solubility–pH profiles of nontreated and charcoal-treated BPA can probably be accounted for in terms of the bound impurities present in the nontreated protein. On the other hand, the extreme broadness of the profile of conventionally defatted protein suggests some irreversible alteration of the protein to accompany the acid-defatting treatment with consequent broadening of the population. This suspicion is reinforced by the results of reciprocal defatting treatments also shown in Figure 1. Charcoal-defatted protein subsequently subjected to acid-defatting conditions shows a pronounced shift of the upper pH part but no shift in the lower part of the profile. Conventionally defatted protein subsequently charcoal defatted shows some sharpening of the profile but the product is substantially broader and the midpoint is at higher pH than in the case of charcoal defatting alone. To a first approximation the broadness

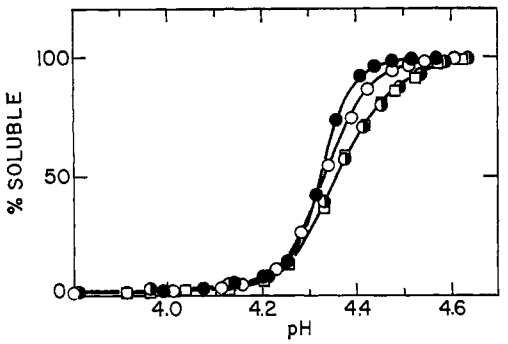

FIGURE 3: Solubility–pH profiles in 3.0 M KCl. (●) Charcoal-defatted BPA; (◑) charcoal-defatted BPA aged at pH 2.835 for 6 days at 2–4°; (□) charcoal-defatted BPA aged at pH 2.402 for 6 days at 2–4°; (○) charcoal-defatted BPA aged at pH 1.930 for 6 days at 2–4°.

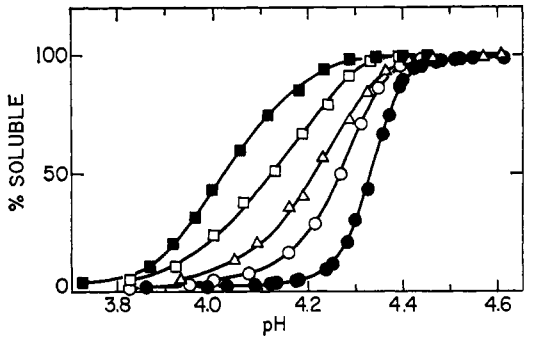

FIGURE 4: Solubility–pH profiles of AD complexes of BPA in 3.0 M KCl. (●) AD_0 (charcoal-defatted BPA); (○) AD_1; (△) AD_2; (□) AD_3; and (■) AD_5.

of the profile of acid-defatted protein can be attributed to two factors, namely (1) incomplete defatting which leads to a shift to the left at low pH and (2) some unknown modification of the protein which leads to a shift to the right at high pH.

The effect of a double charcoal treatment was also studied. BPA was defatted for 2 hr by charcoal treatment in a cold room as described in the Experimental Section. After 30-min centrifugation of the BPA–charcoal mixture, fresh charcoal was added to the supernatant solution (BPA–charcoal, 4:3, dry weight) and the pH of the mixture readjusted to 2.75 by adding dilute HCl. The suspension was stirred for 2 hr in the cold room and then centrifuged for 30 min at 14,000 rpm. After adjustment of the pH to 5.4–5.5, the solubility–pH profile was determined. Except for a very slight difference in midpoint (pH 4.320 *vs.* 4.330) no difference was found in the solubility–pH profiles of twice and singly charcoal-treated BPA.

Instability of Charcoal-Defatted BPA. The observation that subsequent acid defatting of charcoal-defatted protein leads to a substantial shift in the solubility–pH profile in the upper pH region prompted studies of the stability of this protein, using solubility–pH profiles. Even after 20-days storage of charcoal-defatted BPA at pH 5.4–5.5 with presence of 0.03–0.04 M KCl in a cold room (2–4°), the solubility–pH profile was the same as that of fresh charcoal-defatted BPA. However, deionized charcoal-defatted BPA was unstable during storage in the cold room. The solubility–pH profile of charcoal-defatted deionized BPA was unchanged after 0.5-day storage in the cold room but after 2-weeks storage there was a significant change in the solubility–pH profile as shown in Figure 2. There was almost no change in dimer content (~7%) and in SH titer (0.70 mole/mole for aged BPA molecule as compared with 0.73 mole for charcoal-defatted BPA) and there was no detectable change in optical rotatory dispersion parameters (see below). The unusual lability of charcoal-defatted BPA to ageing changes in solution will be the subject of a future paper and evidence will be presented that, at least in part, the changes result from intramolecular sulfhydryl-catalyzed disulfide exchange.

The effect of dialysis on charcoal-defatted BPA was also studied using solubility–pH profiles. Charcoal-defatted BPA (0.035 M KCl) was dialyzed against 4.0 l. of deionized water in a cold room with four changes of precooled deionized water for 4 days using two kinds of pretreated cellophane tubing (circumference, 4.6 and 8.2 cm). Dialyzed charcoal-defatted BPA showed a slight but significant broadening of the solubility–pH profile in the case of large cellophane tubing but not the small tubing. This difference in ageing effect during dialysis might be due to difference in rate of penetration of ions through the two kinds of cellophane tubing.

Figure 3 shows results of ageing charcoal-defatted BPA in solutions of differing pH in the acid region. In all cases low pH causes a broadening of the solubility-pH profile and a shift of the midpoint toward higher pH, the effect being greatest in the pH range 2.4–2.8. This pH range is exactly that in which optimal molecular expansion occurs (in absence of salt) as shown by the viscosity experiments of Yang and Foster (1954). Addition of salt, which markedly represses molecular expansion under these conditions, also reduces the rate of alteration of the solubility–pH profile. This acid-ageing reaction caused no detectable change in the dimer content. Whatever the character of the reaction or reactions responsible for the increased microheterogeneity it seems clear that the rate is enhanced by the very conditions required for optimal release of lipophilic impurities, namely optimal disruption of the three-dimensional molecular structure.

Indications have been obtained in our laboratory that lyophilization of charcoal-defatted BPA may cause some broadening of the solubility–pH profile. For this reason lyophilization was avoided in all of the experiments reported in this paper. In order to avoid population broadening, charcoal-defatted BPA was deionized by passage through the Dintzis column at a relatively rapid flow rate after which the salt concentration was immediately adjusted to the desired concentration, typically 0.10 M KCl. For experiments at very low ionic strength it might be desirable to work with sulfhydryl-blocked BPA. The solubility–pH profile of BPA blocked with iodoacetamide is shown in Figure 2. The slight difference seen there between blocked and unblocked BPA may be due to the fact that blocking was carried out at pH 7.0 and in presence of only 0.035 M KCl. Blocking in presence of at least 0.10 M KCl seems preferable. Clearly, BPA should

not be stored in deionized solution after charcoal defatting.

Effect of Bound Detergent Anions on Solubility–pH Profiles. The effect of stabilizers such as sodium dodecyl sulfate on the N–F transition was studied by Foster and Aoki (1958) and Aoki and Hori (1962). The pH–solubility profiles of AD_1, AD_2, AD_3, and AD_5 are shown in Figure 4. Clearly, bound SDS shifts the midpoint to lower pH and broadens the solubility cruve.

The AD_5 complex, when passed twice through a Dintzis deionizing column, yielded a solubility curve very similar to AD_1 (results not shown). Possibly one or two binding sites for SDS may be stronger than the other binding sites, as in the case of fatty acids (Goodman, 1957, 1958).

The difference in solubility–pH profile between charcoal-defatted BPA and nontreated BPA might be due simply to the difference in bound impurities. The solubility curve of nontreated BPA in 3.0 M KCl (Figure 1) is equivalent to that of $AD_{2.5}$ as interpolated from the curves of Figure 4. The difference between conventionally defatted BPA and charcoal-defatted BPA is more complicated and probably reflects both incomplete defatting and acid-ageing effects during acid treatment, as indicated above. Ample evidence for the latter effect is seen in Figure 3.

It has been reported (Cahn, 1967) that Triton-X 100 is present in Millipore membranes to the extent of 2–3% of the dry weight of the membrane. Therefore, the solubility–pH profile of Millipore-filtered charcoal-defatted BPA was compared with that of ultracentrifuged charcoal-defatted BPA. There was no detectable difference when 75 ml of a 2.5% BPA solution was passed through a fresh membrane. Nevertheless, as a precautionary measure, BPA preparations for titration, optical rotation, difference spectrum, and the later half of the solubility experiments were prepared without using Millipore membranes.

Microheterogeneity of Charcoal-Defatted BPA. It is clear from the solubility–pH profiles presented that charcoal-defatted BPA is far more homogeneous than either nondefatted or conventionally defatted crystallized BPA. There can be no doubt that bound contaminants make an important contribution to the microheterogeneity of these latter preparations. This is in accord with recent findings of McMenamy and Lee (1967) who showed that BPA defatted by a heptane–acetic acid extraction procedure is much more homogeneous than nondefatted protein. In view of this it is pertinent to ask whether the charcoal-defatted protein is microheterogeneous. A detailed study of this question was not made in the present study. On the other hand, it is worth pointing out that a subfraction prepared by the shaking method described under Experimental Section showed significant differences in the midpoint and shape as compared with unfractionated charcoal-defatted BPA. This method has been applied successfully to subfractionate charcoal-defatted BPA in other studies in our laboratory (for example, W. E. Moore and J. F. Foster, manuscript in preparation). It may be true, as McMenamy and Lee (1967) suggest, that their defatting procedure is more effective than the charcoal method and yields a homogeneous preparation. However, we would caution against accepting that conclusion until the improved fractionation method proposed here is applied to such preparations.

Optical Rotatory Dispersion Properties of Charcoal-Defatted BPA. Optical rotatory dispersion data were analyzed using the Moffitt–Yang equation (Moffitt and Yang, 1956) and the Schechter–Blout two-term Drude equation (Schechter and Blout, 1964a,b; Schechter *et al.*, 1964). Estimation of the helix content of BPA from b_0 is complicated by the fact that the measured value depends on scanning range (Sogami *et al.*, 1963). The present data yield $b_0 = -215$ for the range 22–39 $\times 10^3\ \bar{\nu}$ and $b_0 = -250$ for 22–34 $\times 10^3\ \bar{\nu}$ for the most probable λ_0 of 218 mμ. This dependence of b_0 values of BPA on scanning range may result from nonlinearity of the Moffitt–Yang plot due to a Cotton effect around 290 mμ, as discussed below in connection with the application of the Schechter–Blout equation. The Moffitt–Yang equation may be more sensitive to the 290-mμ Cotton effect than the Schechter–Blout equation. Aged charcoal-defatted deionized BPA (aged at pH 8.08 for 86 hr in a cold room without salt) did not show any indication for nonlinearity around 290 mμ in the Schechter–Blout plot. However, the b_0 value of aged BPA still showed a dependence on scanning range, that is, $b_0 = -205$ for 22–39 $\times 10^3\ \bar{\nu}$ and $b_0 = -241$ for 22–34 $\times 10^3\ \bar{\nu}$. The b_0 value for scanning range 22–39 $\times 10^3\ \bar{\nu}$ should be less effected by the Cotton effect around 290 mμ because of a greater contribution of the peptide Cotton effects. Therefore, for comparison of helicity by the b_0 method with those by $[m']_{233}$, A_{225}, or A_{193}, b_0 values obtained by scanning from 22 to 39 $\times 10^3\ \bar{\nu}$ were used.

The next problem relates to the reference b_0 values to be employed for random coil and helix. Leonard and Foster (1963) found that the best value of λ_0 and the associated b_0 for 100% helical poly-L-glutamic acid also depends on the scanning range employed. For the range 18–42 $\times 10^3\ \bar{\nu}$, corresponding closely to the range employed here, they reported b_0 and λ_0 to be $-403°$ and 218 mμ, respectively . According to Riddiford (1966), b_0 of random coiled paramyosine in 7 M guanidine hydrochloride was $+20°$ for $\lambda_0 = 220$ mμ. The helicity of charcoal-defatted deionized BPA in isoionic 0.10 M KCl is ~55% using these parameters. As discussed later, hypothetical values of b_0 for 100% helical and 0% helical BPA were evaluated as -435 and $+33°$, respectively, using the linear relation between b_0 and A_{225} in the acid-expansion region. Using these reference values, the b_0 of $-215°$ corresponds to 53.0% helicity.

For analysis of optical rotatory dispersion data by the Schechter–Blout equation, the data from 22 to 38 $\times 10^3\ \bar{\nu}$ were used because of an assumption made by Schechter and Blout (1964a) in the derivation of their equation, namely that $\lambda \geqslant 260$ mμ ($\bar{\nu} \leqslant 38.5 \times 10^3$). Charcoal-defatted deionized BPA showed some deviation from linearity around 290 mμ (2.002-cm jacketed cell, 0.10 M KCl, 0.0477%) in $[m']((\lambda^2 - 193^2)/193^2)$ *vs.* $225^2/(\lambda^2 - 225^2)$ but HMA or HNA (prepared using the sulfhydryl–disulfide-exchange reaction) did not, as shown in Figure 5. Charcoal-defatted deionized BPA aged at pH 8.08 in a cold room did not show nonlinearity in the Schechter–

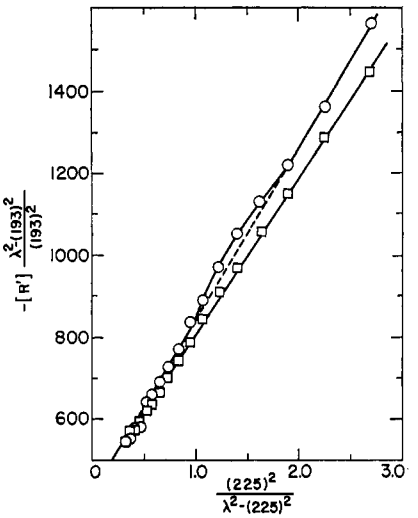

FIGURE 5: Schechter–Blout plots. Ordinate and abscissa are $[R'](\lambda^2 - 193^2)/193^2)$ and $225^2/(\lambda^2 - 225^2)$, respectively. (○) Charcoal-defatted deionized BPA in 0.10 M KCl (H_{225} = 55.1%, H_{193} = 51.9%); (□) conventionally defatted deionized HNA in 0.10 M KCl (H_{225} = 51.6%, H_{193} = 49.7%).

Blout plot. It might be necessary to study circular dichroism before trying to interpret the nonlinearity around 290 mμ. However, we speculate that the nonlinearity in the Schechter–Blout plot may be due to some specific steric arrangement of tryptophyl residues, which is disrupted by ageing above the isoionic pH. A Cotton effect around 290 mμ was also reported for nondefatted BPA by Jirgensons (1962) and by Schellman and Schellman (1956), but was not found by Leonard and Foster (1961) who used conventionally defatted protein. Using the reference values for A_{225} of Schechter and Blout (1964) and data over the range 22–38 × 10^3 $\bar{\nu}$, but ignoring the Cotton effect as indicated in Figure 5, helicities (H_{225}) of charcoal-defatted deionized BPA and conventionally defatted deionized BPA are 55 and 50%, respectively. If only data over the limited range 22–33 × 10^3 $\bar{\nu}$ are used, the corresponding figures for helicity are 60 and 55%. The authors recalculated all of the published optical rotatory dispersion data on BPA from this laboratory using the Schechter–Blout plot. The scanning range of most of the published data was from 579 to 313.2 mμ (17.25–31.9 × 10^3 $\bar{\nu}$). In all cases the helicity (H_{225}) was 54–55%, the same as found in this study using data over this limited range. Therefore, the lower helicity of conventionally defatted deionized BPA as compared with charcoal-defatted or nondefatted BPA appears real and must reflect structural alterations due to the conventional defatting procedure.

It is important to caution on the contribution of nonlinearity around 290 mμ in the analysis of optical rotatory dispersion data of BPA by either the Moffitt–Yang equation or the Schechter–Blout equation. It may be interesting to point out that conventionally defatted HMA showed no nonlinearity around 290 mμ and that the helicity of HMA (H_{225}) using a scanning range of 22–38 × 10^3 $\bar{\nu}$ agreed with the values (54–55%) recalculated from data obtained earlier in this laboratory over the limited range of 17.25–31.9 × 10^3 $\bar{\nu}$.

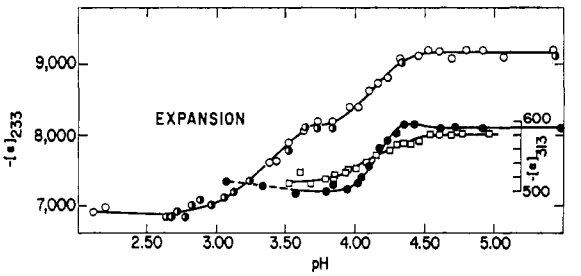

FIGURE 6: Dependence of $[\alpha]_{313}$ and $[\alpha]_{233}$ on pH in 0.10 M KCl. (◑ and ○) Two separate experiments on charcoal-defatted deionized BPA at 233 mμ; (●) charcoal-defatted deionized BPA at 313 mμ; (□) conventionally defatted deionized BPA at 313 mμ. Note separate ordinate scale at right for $[\alpha]_{313}$.

Optical Rotation vs. pH. The dependence on pH of $[\alpha]_{313}$ of charcoal-defatted deionized BPA was compared with that of conventionally defatted deionized BPA in 0.10 M KCl as shown in Figure 6. Clearly, the charcoal-defatted protein yields a much sharper N–F transition than does the conventionally defatted protein and in addition shows unmistakable evidence for a prior transformation near pH 4.5 resulting in an increase in $[\alpha]_{313}$. The difference in pH dependence between charcoal-defatted and conventionally defatted BPA implies a difference in microheterogeneity, in accord with the deduction from the solubility–pH profiles.

The physical meaning of changes in $[\alpha]_{313}$ was interpreted as a reflection of change in tertiary structure by Leonard and Foster (1961). They pointed out that if the Moffitt–Yang equation is taken at face value, the value of the specific rotation near 300 mμ should be independent of helix and coil. We recalculated the wavelength at which changes in secondary structure would not affect the optical rotation using both the Moffitt–Yang equation and the Schechter–Blout equation. Assuming $b_0^H = -630$, $a_0^H/b_0^H = -0.95$ to -1.05, and $\lambda_0 = 212$ mμ, the wavelength of constant rotation would be 296–303 mμ. This ratio of a_0^H/b_0^H was estimated using published data for poly-L-glutamate (Sogami *et al.*, 1963), copoly-L-glutamic acid (Fasman *et al.*, 1964), paramyosine (Riddiford, 1966; Riddiford and Scheraga, 1962), and tropomyosine (Kay and Bailey, 1959). A similar calculation by the Schechter–Blout equation and by their relation $A_{193} = -1.82A_{225} - 782$, showed that optical rotation at 331 mμ would not reflect a change in secondary structure. The mean value of the two estimated wavelengths is about 315 mμ. Therefore, $[\alpha]_{313}$ should not reflect changes in secondary structure provided only helix and coil conformations are present and might mainly reflect changes in tertiary structure. In Figure 6 only the first step of the two transitions, that is the N–F transition, showed a pH-dependent change in $[\alpha]_{313}$.

The specific rotation at the trough of the first Cotton effect, $[\alpha]_{233}$, of charcoal-defatted BPA was studied in 0.10 M KCl as a function of pH as shown in Figure 6. This figure shows clear indication of the two distinct transitions already known. One of the transitions is correlated with the change in $[\alpha]_{313}$ and must correspond to the N–F transition (midpoint pH is 4.15). The second step is presumably due to expansion of BPA (Yang and

TABLE I: Helical Content of Conventionally Defatted BPA and of Charcoal-Defatted BPA under Various Conditions and as Estimated in Various Ways.

BPA	Conditions	Helical Content Estimated from				
		$[m']_{233}$	A_{225}	A_{193}	$-b_0$[a]	$-b_0$[b]
Nontreated	Neutral pH		55.0	52.2	55.1	52.6
Conventionally defatted	Neutral pH	45.4	50.3	47.6	50.8	48.7
Charcoal defatted	Neutral pH	51.2	55.1	51.9	55.6	53.0
Charcoal defatted	F form (pH 3.6–3.9)	43.6	46.7	45.1	48.5	46.6
Charcoal defatted	Expanded (pH <2.7)	34.6	38.0	37.1	37.1	36.3

[a] Using reference values for helix and coil from the literature as described in the text. [b] Using reference values deduced from linear relation between b_0 and A_{225} through the transition region.

Foster, 1954) and presumably can be regarded to first approximation as a helix–coil transition. It might be interesting to point out that the midpoint of the second transition, pH 3.30, shows good agreement with that of the pH for maximum dimerization of BPA in 0.10 M KCl found by Williams and Foster (1960). Helicities of the N form (above pH 4.5), F form (pH 3.6–3.9), and expanded form (below pH 2.7) were 52, 44, and 35%, respectively, as deduced from the values of $[\alpha]_{233}$ using reference values for $[m']_{233}$ for 100% helical and 0% helical states of −14,600 and −1900 as deduced for poly-L-glutamic acid (Tomimatsu *et al.*, 1966).

The b_0–pH profile in 0.10 M KCl is shown in Figure 7 and also reflects the two transitions. Values of a_0 are plotted against corresponding b_0 values in Figure 8. This plot, first suggested by Leonard and Foster (1961), is based again on the Moffitt–Yang equation and provides a test of the extent to which changes other than helix–coil content are involved in a transition. The solid line in Figure 8 has slope of −1.07 ($a_0^H/b_0^H = -1.07$). This slope is quite different from that for conventional defatted deionized BPA, −2.06, as found by Leonard and Foster (1961). Data in the acid-expansion region of charcoal-defatted BPA conform well to the behavior expected for a helix–coil transition of, for example, poly-L-glutamic acid. In the N–F transition range the points deviate drastically from this line as expected. A remarkable feature of the data is the fact that there is a discrete change in the magnitude of a_0 around $b_0 = -200$. In this region the points are numbered and the pH values given in the caption to Figure 8.

Plots of the Schechter–Blout parameters A_{225} *vs.* pH are shown in Figure 7. The A_{225}–pH profiles are very similar to $[\alpha]_{233}$–pH profiles and b_0–pH profiles. Values of H_{225} and H_{193} of the N form (above pH 4.5), F form (pH 3.55–3.90), and expanded form (below pH 2.7) are given in Table I. A plot of A_{193} values *vs.* the corresponding A_{225} values is shown in Figure 9. The dashed line in Figure 9 shows $A_{193} = -1.82A_{225} + 782$ as predicted by the Schechter–Blout equation using parameters for aqueous solutions (Schechter and Blout, 1964a). In the expansion region, charcoal-defatted BPA in 0.10 M KCl conformed to the equation $A_{193} = -1.82A_{225} + 775$. This again supports the contention that acid expansion is equivalent to a helix–coil transition. How-

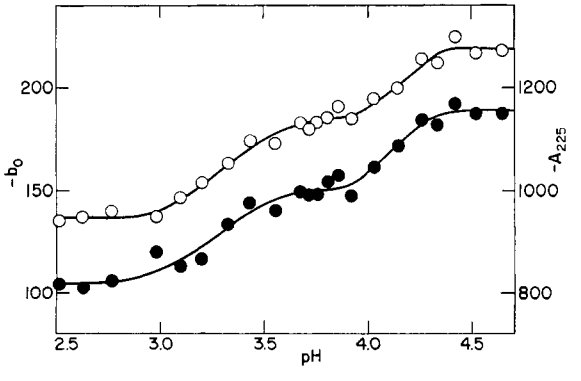

FIGURE 7: Dependence of b_0 (λ_0 = 218 mμ) and of A_{225} on pH, charcoal-defatted deionized BPA in 0.10 M KCl. (○) b_0 (left ordinate scale); (●) A_{225} (right-ordinate scale).

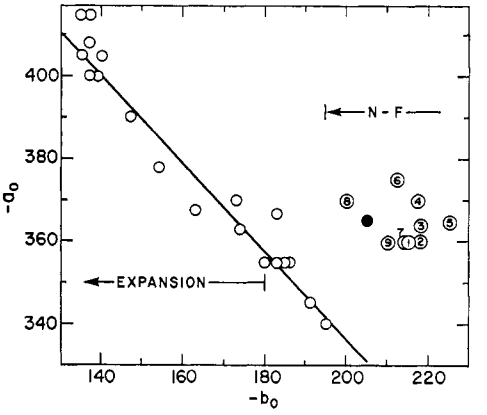

FIGURE 8: Relation between a_0 and b_0, charcoal-defatted deionized BPA in 0.10 M KCl. Unmarked circles correspond to pH values of less than 4.00, covering the range of expansion and the plateau range in, for example, Figures 6 and 7. The numbered circles correspond to: (1) pH 5.498, (2) pH 4.792, (3) pH 4.640, (4) pH 4.515, (5) pH 4.418, (6) pH 4.330, (7) pH 4.253, (8) pH 4.139, and (9) pH 5.410. The shaded circle corresponds to readings at pH 5.465 on a sample of charcoal-defatted deionized BPA aged at pH 8.1 for 4 days at 2°. The slope of the solid line is −1.07, "N–F" and "expansion" indicate ranges of N–F transition and acid expansion, respectively.

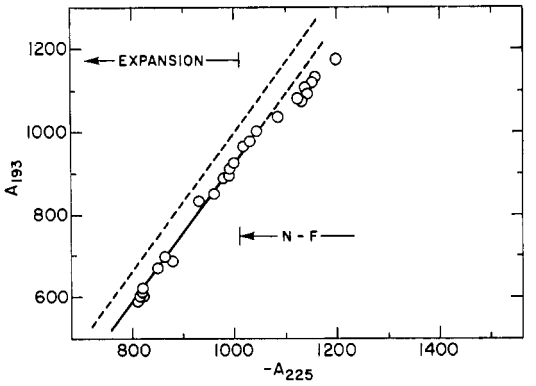

FIGURE 9: Relation between A_{193} and $-A_{225}$ for charcoal-defatted BPA in 0.10 M KCl. Broken line is the published relation of Schechter and Blout, $A_{193} = -1.82A_{225} - 782$. The solid line and dashed extension shows relation between A_{193} and A_{225} through the acid-expansion region, namely $A_{193} = -1.82A_{225} - 775$. "N–F" and "expansion" show ranges of N–F transition and acid expansion.

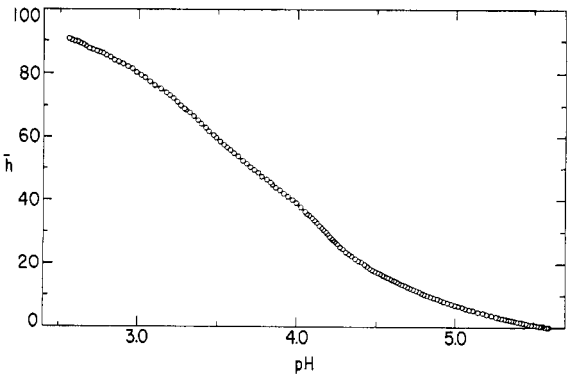

FIGURE 11: Hydrogen ion titration curve of charcoal-defatted and deionized BPA in 0.10 M KCl, for the range below the isoionic pH. Graph shows mean number of hydrogen ions bound per molecule of BPA measured from the isoionic pH and assuming the molecular weight of BPA to be 66,000.

ever, A_{193} *vs.* A_{225} in the N–F transition region was also complicated as in the case of a_0 *vs.* b_0 (Figure 8). According to considerations of Schechter *et al.* (1964) on the effect of dielectric constant on A_{225} and A_{193}, the N–F transition would be considered equivalent to the transfer of peptide from water to a nonpolar environment. The discrete change in magnitude of a_0 around $b_0 = -200°$ in Figure 8 is equivalent to a decrease in the magnitude of Σa_0^R which, according to Tanford (Tanford, 1962; Tanford *et al.*, 1960), could be interpreted as due to transfer of peptide residues from water to a nonpolar environment. However, these interpretations are in contradiction with other evidence that the N–F transition involves an opening of the globular structure with increased exposure of peptide residues (Foster, 1960). Schuster (1965) and Tomimatsu *et al.* (1966) observed that b_0 remains essentially constant and that a_0 values become more negative with aggregation of helical poly-L-glutamic acid and interpreted the change in a_0 as due to extensive lateral overlapping and close association of helical chains. In terms of these findings there appears to be no incompatability of the present results with the picture of the N–F transition as a separation of intramolecular substructures (Foster, 1960) or the opening of a crevice (Laskowksi, 1966). In fact, since the opening of the crevice is evidently the second of the two steps in the N–F transition (Herskovits and Laskowski, 1962), it is reasonable to assume that this event is responsible for the gross discontinuity in Figure 8.

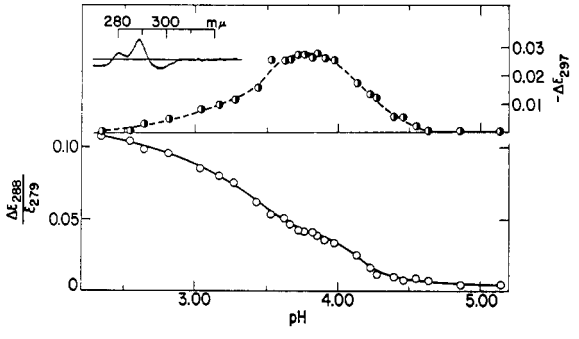

FIGURE 10: Acid-difference spectrum of charcoal-defatted BPA and its dependence on pH. Measurements were in 0.10 M KCl *vs.* a reference solution of pH 5.56 in all cases. In the upper left-hand corner is shown an example (at pH 3.98) of the difference spectra obtained. (○) $\Delta\epsilon_{288}/\epsilon_{279}$, left-hand ordinate scale; (◑) $\Delta\epsilon_{297}$, right-hand ordinate scale.

A plot of $[\alpha]_{233}$ *vs.* the A_{225} parameter of the Schechter–Blout equation yielded a good linear relationship corresponding to the equation $[\alpha]_{233} = 6.9A_{225} - 1.2 \times 10^3$. Assuming A_{225} values for 0% helix and 100% helix as -60 and -2050, respectively (Schechter and Blout, 1964a), hypothetical $[m']_{233}$ values for 100% helical BPA and for 0% helical BPA were estimated as -1.39×10^4 and -1.45×10^3, respectively. These values are very similar to $[m']_{233}$ values reported for helical and coil forms of poly-L-glutamic acid by various workers. Using these estimated values for $[m']_{233}$ of helix and coil, the helicity of charcoal-defatted deionized BPA in 0.10 M KCl (isoionic) was 55–56% instead of the 52% based on Tomimatsu's reference values (above).

Similarly, observed b_0 values were plotted against A_{225} yielding a linear relationship throughout both the N–F transition region and acid-expansion region corresponding to $b_0 = 0.24A_{225} + 57$. Using reference values of A_{225} of Schechter and Blout (1964a), b_0 values for 100% helical BPA and for 0% helical BPA were estimated as -435 and $+33°$, respectively, at $\lambda_0 = 218$ mμ. The value for 100% helix is very close to the $-423°$ of helical poly-L-lysine ($\lambda_0 = 218$ mμ) and $-442°$ of poly-γ-benzyl-L-glutamate $\lambda_0 = 219$ mμ) (Leonard and Foster, 1963). The helicity of isoionic charcoal-defatted BPA ($b_0 = 215°$) is 53%, using these reference values (see Table I).

Acid-Difference Spectrum. Isoionic charcoal-defatted deionized BPA in 0.10 M KCl (pH 5.563) and acidified BPA in 0.10 M KCl were placed in the sample and reference beams, respectively, of the Cary 14 spectrophotometer. One example of a difference spectrum so obtained is given in the left upper part of Figure 10. The shape of the spectrum is closely similar to that found earlier under similar conditions for conventionally defatted BPA. It is also typical of the "tyrosyl" difference spec-

tra which have become well known in the protein field. A somewhat novel feature of this spectrum is the trough near 297 mμ which first increases in magnitude, then decreases as the pH is lowered (Figure 10, upper curve). On the other hand, the peak at 288 mμ increases progressively but with a distinct hint of two steps corresponding to the N–F transition and expansion, respectively. Whether the trough is due to the tyrosyl chromophore or to tryptophan is not at all clear. The shape is somewhat different from the difference spectrum attributed to tryptophyl residues in detergent complexes of BPA (Bigelow and Sonenberg, 1962; Ray *et al.*, 1966). Whatever the origin of this feature of the acid-difference spectrum it clearly provides a dramatic empirical means for distinguishing the N–F transformation from the subsequent expansion.

At the end of the N–F transition, the magnitude of $\Delta\epsilon_{288}/\epsilon_{279}$ is about 40% of that at pH 2.3. According to the interpretation of Williams and Foster (1959) this is due to exposure of tyrosyl residues from a hydrophobic to an aqueous environment. In more precise terms, the process probably corresponds to the opening of a crevice as postulated by Herskovits and Laskowski (1962). The value of $\Delta\epsilon_{288}/\epsilon_{279}$ for charcoal-defatted BPA in 0.10 M KCl at pH 2.34 is exactly equal to that of conventionally defatted deionized BPA under the same conditions (Vijai, 1966).

Hydrogen Ion Titration in 0.10 M KCl. It was of interest to determine the pH titration behavior of charcoal-defatted BPA because of the evidence presented above that its N–F transition is much sharper than that of any of the preparations previously studied. The number of bound hydrogen ions per BPA molecule is given as a function of pH in Figure 11 and shows a slight but significant indication for two steps in the protonation corresponding to the N–F transition and expansion. The data of Vijai and Foster (1967) also exhibit some indication of two steps in the titration curve for conventionally defatted deionized BPA. Using electrophoretic mobility data of conventionally defatted deionized BPA in 0.10 M NaCl at 25° (Vijai and Foster, 1967), the authors estimated pK_{int} and the maximum number of carboxyl groups of charcoal-defatted deionized BPA in 0.10 M KCl, as in the case of Vijai and Foster (1967). Charcoal-defatted deionized BPA showed a curve for $\bar{h}/(a_{H^+})\exp(\epsilon\psi/kT)$ *vs.* $\bar{h}$ similar to that of conventionally defatted deionized BPA (Vijai and Foster, 1967). Values of pK_{int} for the initial 50 carboxyl groups in the N form and for all of the groups in the F form were 4.30 and 4.00, respectively, compared with the earlier values of 4.25 and 4.00. The pK_{int} values are a little low as compared with numerous reported values for normal carboxyl groups.

The total number of titratable carboxyl groups obtained by extrapolation of the present data is 102/BPA molecule, assuming a molecular weight of 66,000 and the number of protonated carboxyl groups in the isoionic solution to be 3.0 (Vijai and Foster, 1967). This number is very close to the value of 98–100 expected on the basis of the best available amino acid composition data (Spahr and Edsall, 1964). However, the value of 102 is significantly smaller than the value of 108 found by Vijai and Foster (1967). This raises the possibility that some deamidation may occur during the prolonged acid treatment in conventional defatting, as has been found recently in the case of cytochrome C (Flatmark, 1966).

The above experiments on charcoal-defatted deionized BPA are mainly of a static character. It would be of interest to study dynamic properties of charcoal-defatted BPA such as deuterium–hydrogen exchange or the reactivity of buried disulfide bonds as functions of pH, salt concentration, fatty acid, or detergent to complete our understanding of the difference between charcoal-defatted BPA and conventionally defatted BPA.

Added in Proof

Attention is called to a recent paper by Katz and Denis (1967) which also demonstrates the unusual lability of charcoal-defatted BPA reported in this paper.

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