Removal of Fatty Acids from Serum Albumin by Charcoal Treatment

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SUMMARY

The fatty acid contents of 26 different serum albumin preparations representing different species and obtained from various commercial sources have been determined. Some samples had surprisingly little fatty acid contamination, but it was found that other samples contained between 2 and 3 moles of acid per mole of protein, in confirmation of earlier reports. Treatment of these samples with charcoal at low pH resulted in the virtually complete removal of fatty acids. The conditions for such treatment were investigated as a function of the type of fatty acid, pH, and the amount of charcoal required. Charcoal treatment removed at least 99% of albumin-bound radioactive fatty acids under optimal conditions, whereas the amount of protein adsorbed onto charcoal was 4.9%. The protein that remained in solution following separation of the charcoal appeared to be native, as judged by a number of criteria, including analytical ultracentrifugation, optical rotatory dispersion, the ability to bind fatty acids, and the ability to bind the fluorescent dye 8-anilinonaphthalene-1-sulfonic acid, as well as several other fluorescence parameters. Fluorescence spectra of human serum albumin samples indicated that impurities are sometimes present which can be removed by charcoal at neutral pH. A possible explanation for the gentleness of charcoal treatment is offered. Acid-charcoal treatment is a much more rapid method of removing lipid impurities than other methods previously described.

Analysis of a sample of HSA by Saifer and Goldman (5), who used gas-liquid chromatography, showed that a variety of fatty acids was present, the most prominent being oleic, palmitic, and linoleic acids. The exact composition of the lipid impurity in albumin probably varies from sample to sample, but it is generally agreed that current methods of preparing this protein result in the presence of some such impurity. Foster (6) pointed out that not enough attention has been paid to such contamination, and that it is not clear whether the fatty acids in BSA and HSA have influenced the results of published studies on the physical properties of these proteins. For instance, the presence of FFA would tend to influence the electrophoretic mobility of albumin, and could also affect its conformation. Binding studies involving albumin could be drastically altered depending on whether the ligand of interest were to compete for a site occupied by fatty acid contaminants; examples of such a situation are the binding of tryptophan by HSA (7), and, of course, the binding of other fatty acid anions (8). Certain metabolic studies with the use of complexes of albumin with radioactive fatty acids (e.g. Reference 9) require defatted albumin as starting material, since the presence of contaminating (“endogenous”) acids would lower to an uncertain degree the specific radioactivity. For these and other reasons, it is desirable to have a convenient method of removing fatty acid impurities.

Two procedures described for this purpose are those of Goodman (10) and Williams and Foster (11). In Goodman's method, dry albumin is extracted with a mixture of isooctane and glacial acetic acid for at least 6 hours, washed with isooctane, extracted again with isooctane-acetic acid, washed repeatedly with isooctane, dried in a vacuum, and, finally, dialyzed exhaustively against buffer. In the method of Williams and Foster, the concentrated albumin solutions are kept at pH 2.9 for 2 to 3 days, during which time lipids form a separate phase and can be removed by centrifugation. However, these authors subsequently stated, “It is noteworthy that this procedure for removal of the lipid material is not always 100% efficient” (4). Since both methods are time-consuming and documentation of their effectiveness is incomplete, it was decided to investigate the effect of activated charcoal on the FFA content of albumins. The present communication reports that charcoal treatment at low pH is a rapid and effective method of defatting albumins from a variety of sources. The method seems to be effective for different fatty acids, and does not result in any gross structural changes in the protein which are detectable at neutral pH by

It has been known for many years that serum albumin preparations contain variable amounts of lipid impurity (1, 2); this appears to be true not only of samples prepared by fractionation but also of crystalline human serum albumin and bovine serum albumin. The bulk of the impurity appears to consist of free (i.e. unesterified) fatty acids, and infrared spectroscopy of the material isolated from BSA1 has indicated that stearate is present (3, 4). Williams and Foster (4) suggested that other homologous saturated fatty acids are probably present, and Bro and Sturtevant (3) postulated that dinonyl ketone was present as well.

1 The abbreviations used are: BSA, bovine serum albumin; HSA, human serum albumin; FFA, free fatty acid or acids; ANS, 8-anilinonaphthalene-1-sulfonic acid.
analytical ultracentrifugation, optical rotatory dispersion in the far ultraviolet, or fluorescence techniques.

**EXPERIMENTAL PROCEDURE**

**Materials**—The commercial sources and lot numbers of the albumin samples are generally indicated as they occur in the text, figures, and tables. Otherwise, Armour BSA, Fraction V, Lot M12005 or B28308, was used. Fatty acids were obtained from the following sources: Eastman, octanoic acid; Hormel Institute, crystalline lauric, myristic, palmitic, oleic, and stearic acids; Nuclear-Chicago, oleic acid-1-14C; New England Nuclear, palmitic acid-1-14C and stearic acid-1-14C. The radioactive fatty acids, which had been purified by extraction into alkaline ethanol followed by acidification and re-extraction into isooctane (9) were found by gas-liquid chromatography to be at least 96.5% pure. The activated charcoals used were Darco M, Lot DXL-0-2881, obtained from Atlas Chemical Industries, and Norit A, Lots M12005 or B23809, was used. Fatty acids were obtained of 0.2% purity. The activated charcoals used were Darco M, Lot DXL-0-2881, obtained from Atlas Chemical Industries, and Norit A, Lots M12005 or B23809, obtained from Fisher Chemical Company. The charcoals were washed with distilled water, filtered with a Buchner funnel, and allowed to dry at room temperature. 8 Anilinonaphthalene-1-sulfonic acid was obtained as the sodium salt from the J. T. Baker Chemical Company, Phillipsburg, New Jersey, and was crystallized twice as the magnesium salt (12).

Complexes of BSA with fatty acids were made simply by adding FFA in a slight excess of aqueous KOH to solutions of BSA which had previously been treated with charcoal.

Protein concentration was measured by the method of Lowry et al. (13) or from the A279 as determined with a Beckman DU spectrophotometer. The assumed extinctions at 279 mp for 1% solutions of BSA and HSA were 6.67 and 5.30, respectively (14, 15).

**General Procedure for Removal of Fatty Acids from Serum Albumin**—Albumin (7.0 g) was dissolved in 70 ml of distilled water at 23°C. Darco (3.5 g) was mixed into the solution, and the pH was lowered to 3.0 by the addition of 0.2 ml HCl. The solution was then placed in an ice bath and mixed magnetically for 1 hour. Charcoal was then removed by centrifugation at 20,200 g for 20 min in a Sorvall RC1 centrifuge with an SS 34 rotor at 2°C. The clarified solution was then brought to pH 7.0 by the addition of 0.2 ml NaOH.

**Assay of FFA**—A modification of the procedures of Dole (16) and Trout, Estes, and Friedberg (17) was briefly described by Spector, Steinberg, and Tanaka (9). As used in this study, the method is as follows. Sample (1.0 ml) containing 30 to 70 mg of albumin and 0 to 3 µeq of FFA was shaken with 5.0 ml of extraction mixture in a glass-stoppered test tube and allowed to stand for 15 min. The extraction mixture contained isopropyl alcohol-isoctane-1 N H2SO4 (40:10:1). Isooctane (3.0 ml) and 2.0 ml of 0.1 N H2SO4 were then added to the test tube, and the contents were shaken. The upper phase was transferred to another glass-stoppered test tube containing 5.0 ml of 0.1 N H2SO4 and the contents were shaken. The isooctane phase (3.0 ml) was transferred to a conical centrifuge tube containing 1 ml of a titration mixture, consisting of 0.002% Nile blue A in 90% (v/v) ethanol. The FFA was then titrated with 0.02 N NaOH to a light pink end point, with mixing being effected by a stream of CO2-free nitrogen. The titrations permitted the determination of the FFA content of samples with a precision of ±0.03 mole of FFA per mole of albumin. However, from experiments with radioactive fatty acids (see below), it appears that the titrimetric assay occasionally gave slightly high values, since albumin samples containing negligible radioactivity seemed to have 0.02 to 0.06 µeq of titratable acid per mole of albumin. It is presumed that this slight artifact may be due to a degradation product from protein formed during the vigorous shaking of the acid-isoctane extraction, especially since the protein concentration was high.

Radioactivity was measured with a Packard Tri-Carb scintillation counter. To determine FFA content of albumin samples, the extraction procedure was carried out as above and 3.0 ml of the final isoctane phase were taken to dryness under a stream of N2 in a counting vial. The radioactivity of the residue was determined after the addition of 10 ml of toluene containing phosphor (9).

Optical rotatory dispersion was determined with a Cary model 60 spectropolarimeter with the cell compartment maintained at 27°C. A cylindrical cell with a 0.100-cm path length was used for all measurements.

Fluorescence was measured with an Amino-Bowman spectrophotofluorometer fitted with an emission monochromator grating blazed for maximum transmission in the first order at 300 mp. Excitation energy was passed through a horizontally oriented polarizing filter in order to reduce scattered light signal (18), as well as to attenuate the light so as to avoid photodecomposition of sample. Calibration of the detector system, which utilized an RCA 1P28 photomultiplier tube, was carried out as previously described (19), and showed that the detector response, expressed in terms of relative quanta, was essentially flat from 280 to 400 mp. The spectra of Fig. 10 can therefore be taken as "true" emission curves without correction. The other emission spectra (Fig. 9) are corrected for detector response with the use of the calibration data.

**RESULTS**

**FFA Content of Albumin Preparations**—Albumin samples derived from different species were obtained and their FFA contents measured. The results, shown in Table I, indicate great variation in FFA content, ranging from less than 0.1 to more than 2 eq per mole of albumin. Samples of HSA which were examined were particularly high in FFA content, and three of the four crystalline BSA samples contained close to 1 eq of FFA. The wide variation in FFA content shows that no assumption can be made concerning the lipid content of any given sample. When each sample was treated with charcoal (Darco M) at pH 3.0 under the conditions described under "Experimental Procedure," the FFA content was reduced to below 0.1 eq per mole of protein, as also shown in Table I. In a sense, the results of this table anticipate the data of the following sections, since the conditions used for charcoal treatment were arrived at by systematically studying the effects of pH, amount of charcoal required, and the amount of contact time needed. These results are shown below.

**Effect of pH**—Initial attempts to remove FFA from albumin by charcoal treatment in solutions of pH 5 to 8 were only partially successful. This is not surprising, in view of the extreme tightness with which these acids are bound; Goodman (8) found the first two association constants for binding of stearic, palmitic, and oleic acids were of the order of 108 liters per mole. The...
Effect of pH on the release of FFA was investigated with an albumin-palmitate complex, as shown in Fig. 1. While charcoal reduced the FFA content per mole of albumin from 3.6 to 1.7 even at neutral pH, release of the last mole of palmitate was not effected until the pH was lowered below 4. The midpoint of the curve of Fig. 1 is at pH 4.5, so it seems probable that both the molecular unfolding of albumin centered at pH 4 and the protonation of palmitate at pH 5 are important in rendering the fatty acid accessible to charcoal.

Removal of Various Fatty Acids—Complexes of BSA with various other FFA were prepared and then treated with different acids. The effect of pH on the release of FFA was investigated with an albumin-palmitate complex, as shown in Fig. 1. While charcoal reduced the FFA content per mole of albumin from 3.6 to 1.7 even at neutral pH, release of the last mole of palmitate was not effected until the pH was lowered below 4. The midpoint of the curve of Fig. 1 is at pH 4.5, so it seems probable that both the molecular unfolding of albumin centered at pH 4 and the protonation of palmitate at pH 5 are important in rendering the fatty acid accessible to charcoal.

\[ \text{FFA content of albumin samples} \]

<table>
<thead>
<tr>
<th>Preparations</th>
<th>Type</th>
<th>Lot No.</th>
<th>Before charcoal treatment</th>
<th>After charcoal treatment*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bovine</td>
<td>Crystalline</td>
<td>11</td>
<td>1.05 ± 0.07</td>
<td></td>
</tr>
<tr>
<td>Pentex</td>
<td>Crystalline</td>
<td>1x4</td>
<td>0.66 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Armour</td>
<td>Crystalline</td>
<td>X69508</td>
<td>0.81 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Armour</td>
<td>Crystalline</td>
<td>A69805</td>
<td>0.88 ± 0.01</td>
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</tr>
<tr>
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<td>Fraction V</td>
<td>31-A</td>
<td>0.18 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Armour</td>
<td>Fraction V</td>
<td>W18602</td>
<td>0.49 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Armour</td>
<td>Fraction V</td>
<td>B24111</td>
<td>0.38 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Armour</td>
<td>Fraction V</td>
<td>M12005</td>
<td>0.31 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Human</td>
<td>Crystalline</td>
<td>Hx1</td>
<td>0.42 ± 0.02</td>
<td></td>
</tr>
<tr>
<td>Pentex</td>
<td>Crystalline</td>
<td>9</td>
<td>0.74 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Pentex</td>
<td>Crystalline</td>
<td>Hx3</td>
<td>0.69 ± 0.01</td>
<td></td>
</tr>
<tr>
<td>Pentex</td>
<td>Crystalline</td>
<td>16</td>
<td>0.06 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Nutritional Biochemicals</td>
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<td>2.50 ± 0.03</td>
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</tr>
<tr>
<td>Nutritional Biochemicals</td>
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<td>6941</td>
<td>1.83 ± 0.01</td>
<td></td>
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<tr>
<td>Nutritional Biochemicals</td>
<td>Fraction V</td>
<td>7514</td>
<td>2.20 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>American Red Cross</td>
<td>Fraction V</td>
<td>1892</td>
<td>2.36 ± 0.05</td>
<td></td>
</tr>
<tr>
<td>Canine</td>
<td>Fraction V</td>
<td>C32</td>
<td>1.12 ± 0.08</td>
<td></td>
</tr>
<tr>
<td>Pentex</td>
<td>Fraction V</td>
<td>C37</td>
<td>0.10 ± 0.03</td>
<td></td>
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<tr>
<td>Equine</td>
<td>Fraction V</td>
<td>E51</td>
<td>0.18 ± 0.00</td>
<td></td>
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<td>Nutritional Biochemicals</td>
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<td>5006</td>
<td>0.43 ± 0.06</td>
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<tr>
<td>Rabbit</td>
<td>Fraction V</td>
<td>R52</td>
<td>0.15 ± 0.00</td>
<td></td>
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<tr>
<td>Nutritional Biochemicals</td>
<td>Fraction V</td>
<td>3517</td>
<td>0.14 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Pentex</td>
<td>Fraction V</td>
<td>R51</td>
<td>0.10 ± 0.00</td>
<td></td>
</tr>
<tr>
<td>Porcine</td>
<td>Fraction V</td>
<td>J301</td>
<td>0.10 ± 0.03</td>
<td></td>
</tr>
<tr>
<td>Nutritional Biochemicals</td>
<td>Fraction V</td>
<td>1085</td>
<td>0.08 ± 0.01</td>
<td></td>
</tr>
</tbody>
</table>

* See general procedure as detailed under "Experimental Procedure."

b Calculated on the basis of a molecular weight of 69,000.

![Fig. 1. Effect of pH on the removal of palmitate from BSA.](http://www.jbc.org/)

**Removal of Various Fatty Acids—**Complexes of BSA with various other FFA were prepared and then treated with different acids. The effect of pH on the release of FFA was investigated with an albumin-palmitate complex, as shown in Fig. 1. While charcoal reduced the FFA content per mole of albumin from 3.6 to 1.7 even at neutral pH, release of the last mole of palmitate was not effected until the pH was lowered below 4. The midpoint of the curve of Fig. 1 is at pH 4.5, so it seems probable that both the molecular unfolding of albumin centered at pH 4 and the protonation of palmitate at pH 5 are important in rendering the fatty acid accessible to charcoal.

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Fig. 2. Removal by charcoal of fatty acids bound to BSA. The amount of fatty acid remaining after treatment with charcoal at pH 3.0 and 2°C is plotted against the amount of charcoal added. In each case, a BSA preparation was used which was previously treated with charcoal to remove endogenous FFA (see “General Procedure for Removal of Fatty Acids from Serum Albumin”) and then charged with the indicated fatty acid anion in the form of its potassium salt. The charcoal was removed by centrifugation after 1 hour of mixing time. The points labeled control represent the amount of FFA present before charcoal treatment, and are distinguished from the other points at charcoal = 0, which are samples which have lost some FFA solely as a result of standing for 1 hour at pH 3.0 and centrifugation.

The amount of contact time required for different samples might reflect differences in the types of fatty acids bound; however, this is not clearly established. At any rate, a contact time of 1 hour appears to suffice for all commercial preparations tested (Table I), and was adopted for the general procedure described under “Experimental Procedure.”

Other Variables—Experiments similar to those of Fig. 2 were carried out in the presence of 0.5 M NaCl with no detectable difference in results, thus suggesting that the ionic strength of the solutions is not a particularly important factor under these conditions. Usually, charcoal was applied to 4 to 6% albumin solutions, but other experiments with more dilute solutions (down to 1%) showed no significant decrease in the effectiveness of charcoal treatment.

Although the experiments reported here were done with Darco M, a limited number of studies such as those of Fig. 1 were done with Norit A and Nuchar-C190-N charcoals. No significant difference was noted among the three charcoals in their capacity to remove FFA from BSA. All three types of charcoal are derived from wood or pulp (20), but they are activated by different processes.

It was noted that the centrifuged charcoal particles, in the presence of serum albumin, tended to become resuspended with time, probably because of charge repulsion, since it is likely that the particles are coated with albumin which has a net positive charge at pH 3. For this reason, the protein supernatants were always decanted immediately after centrifugation.

Protein Recovery—The optical density at 280 nm of BSA solution treated with a weight of charcoal equal to that of the protein showed a 4.9% decrease, suggesting that no more than this amount of protein was actually adsorbed by the carbon. In the general procedure given above, however, the protein recovery is usually only 80% because of occlusion of protein solution in the charcoal pellet.

Ultracentrifugation of Charcoal-treated Albumin—Work of Williams and Foster (4) and Hartley, Peterson, and Sober (21) showed that dimerization of albumin occurred at low pH. Since charcoal treatment was performed at pH 3.0 to remove fatty acids in this study, it seemed possible that some degree of aggregation could have been induced. For this reason, samples of treated and untreated BSA (from the same crystalline preparation) were examined in the analytical ultracentrifuge with the two solutions in separate parts of a double-sector cell. The results (Fig. 5) showed no evidence of aggregation, and the original homogeneity of the preparation was undisturbed.

Optical Rotatory Dispersion—The cotton effects of proteins in the far ultraviolet region, 190 to 240 nm, have been found to be sensitive indicators of protein conformation (22). The specific rotation of albumin in the negative trough at 233 nm has been used to show that when albumin is oxidized it loses much of its helical structure (22). The rotatory dispersion of BSA was examined before and after charcoal treatment (Fig. 6). No decrease in the magnitude of the 233 nm trough can be seen, and one can reasonably conclude that there was no gross disruption of helicity during the acid-charcoal procedure. The observed rotation actually seems slightly greater with the treated protein at neutral pH, although the magnitude of the difference is probably within the limits of experimental error.
TABLE II
Removal of radioactive fatty acids from bovine serum albumin
Charcoal treatment was carried out at 2°C. Except for the controls, all samples were kept at pH 3.0 for the stated times and then centrifuged at 20,000 × g. A molecular weight of 69,000 was assumed for BSA.

<table>
<thead>
<tr>
<th>Sample and treatment</th>
<th>Activity FFA</th>
<th>FFA removeda</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm/mole protein</td>
<td>mole/mole protein</td>
</tr>
<tr>
<td>BSA-oleate-1-14C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Control</td>
<td>48,000</td>
<td>3.85</td>
</tr>
<tr>
<td>2. 1 mg of Darco M per mg of protein, 30-min contact</td>
<td>1,040</td>
<td>0.01</td>
</tr>
<tr>
<td>3. Same as 2, but 150-min contact</td>
<td>754</td>
<td>0.01</td>
</tr>
<tr>
<td>BSA-palmitate-1-14C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4. Control</td>
<td>1,316,000</td>
<td>3.40</td>
</tr>
<tr>
<td>5. Same as 2</td>
<td>1,880</td>
<td>0.00</td>
</tr>
<tr>
<td>6. Same as 3</td>
<td>1,255</td>
<td>0.00</td>
</tr>
<tr>
<td>BSA-stearate-1-14C</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S1. Control</td>
<td>57,500</td>
<td>2.07</td>
</tr>
<tr>
<td>S2. No charcoal, 120-min contact</td>
<td>56,600</td>
<td>2.04</td>
</tr>
<tr>
<td>S3. 0.15 mg of Darco M per mg of protein, 120-min contact</td>
<td>28,300</td>
<td>1.02</td>
</tr>
<tr>
<td>S4. Same as S3, but 0.33 mg of Darco M per mg of protein</td>
<td>6,270</td>
<td>0.23</td>
</tr>
<tr>
<td>S5. Same as S3, but 0.67 mg of Darco M per mg of protein</td>
<td>1,695</td>
<td>0.06</td>
</tr>
<tr>
<td>S6. Same as S3, but 1.0 mg of Darco M per mg of protein</td>
<td>486</td>
<td>0.02</td>
</tr>
<tr>
<td>S7. Same as S3, but 1.3 mg of Darco M per mg of protein</td>
<td>323</td>
<td>0.01</td>
</tr>
</tbody>
</table>

a Calculated from the specific radioactivity of the added FFA.

It is known that long chain acids stabilize albumin against the denaturing effect of alkali (23), and there was a possibility that the complete removal of endogenous FFA might make BSA particularly susceptible to conformational change at high pH. However, the rotatory dispersion of crystalline BSA in the far ultraviolet at pH 10.1 showed no difference between the treated and untreated samples (Fig. 7). On the other hand, when 3 moles of palmitate per mole of protein were present, a slightly larger levorotation was observed. These results would seem to suggest that, at pH 10.1, palmitate has prevented some conformational change in BSA which took place to the same extent in the two solutions (untreated and charcoal-treated) to which palmitate was not added.

Fluorescent Probe Experiments—In a further attempt to show a change in albumin structure due to charcoal treatment, the "fluorescent probe" method (24) was used, with the use of the dye, ANS. The principle is simply that dyes such as ANS, when bound to certain proteins, possess a strong fluorescence which is sensitive to changes in protein conformation (25-28). This method has the advantage that it can be used with Fraction V samples, which are not homogeneous protein preparations, since the ANS fluorescence arises from the specific binding to albumin (29). The fluorescence of ANS bound to different samples of BSA shows considerable variation with pH (Fig. 8). The chief features are a decrease in ANS fluorescence beginning at about pH 5 and reaching a minimum at about pH 3.9 to 4.0, a sharp peak at pH 3.35, followed by a decrease below pH 3 to a level which is less than that at neutral pH. In the alkaline region, ANS fluorescence tends to increase and then suddenly fall near pH 10.8. Fig. 8 shows that there are distinct differences in the pH dependence curves for ANS bound to different BSA samples. In the case of the untreated BSA, containing 0.33 mole of FFA per mole of protein, the enhance-
ment of ANS fluorescence seems to occur at a higher pH, 9.7, than in the case of the charcoal-treated BSA, in which the fluorescence increases at pH 8.7. Furthermore, it appears that the decrease in ANS fluorescence on going from neutral to acid pH occurs earlier in the latter case, and the minimum of ANS emission occurs some 0.2 pH unit higher than for the untreated BSA. This difference, however, is most likely related to the different FFA content. Thus, Curve C, for which charcoal-treated BSA containing added palmitate (3 moles per mole of protein) was used, shows the marked stabilising effect of the fatty acid on the alkaline transition, although there is a gradual rise in emission intensity of the ANS. Below pH 4, turbidity due to precipitation of palmitic acid rendered the experimental data somewhat uncertain, but again there is a sharp peak in the neighborhood of pH 3.4. It should be noted that, in Fig. 8, the same relative scale for fluorescence intensity is used for the three curves, and it can be seen that, at neutral pH, the fluorescence of ANS has the same intensity for each sample. Because ANS emission is extremely sensitive to the environment near the dye, one can conjecture that the binding site for ANS must be different from those for the 3 most tightly bound palmitate molecules, since the presence of this FFA does not influence the fluorescence yield of ANS. Also, the fact that charcoal-treated and untreated BSA samples give the same quantum yield for bound ANS is further evidence that structural change has not occurred.

The sharp peak in ANS fluorescence which occurs at pH 3.35 is of particular interest and deserves additional comment. It is in this region that serum albumin is known to have unfolded, exposing to the solvent large numbers of hydrophobic residues which were originally in the interior of the protein (6, 30). Williams and Foster (4) found, for instance, that the optimal pH

Fig. 5. Ultracentrifuge schlieren patterns of Armour crystalline BSA lot A69805 before (lower curve) and after (upper curve) charcoal treatment. FFA content was 0.90 eq per mole of protein before, and 0.01 eq after, treatment. The preparation was treated essentially as described under “General Procedure for Removal of Fatty Acids from Serum Albumin,” with buffer, 0.1 M potassium phosphate at pH 6.8, and protein concentration, 0.5%. The picture was taken 32 min after a rotor speed of 56,100 rpm had been reached; the temperature was 23°.

Fig. 6. Optical rotatory dispersion curve of human serum albumin (Pentex Lot 9) in 0.01 M Tris-Cl buffer, pH 7.0. Before charcoal treatment (dashed line), the preparation contained 0.74 eq of FFA per mole of protein; following treatment (solid line), there were 0.01 eq of FFA present. Protein concentration was 1.0 mg per ml.

Fig. 7. Optical rotatory dispersion curves of bovine serum albumin (Armour Fraction V, Lot M 12005) at alkaline pH. The curve for untreated BSA, containing 0.33 eq of FFA, was identical with the curve obtained for charcoal-treated BSA (solid line), containing 0.01 eq of FFA. The spectrum for charcoal-treated BSA to which 3.0 eq of potassium palmitate were added is also shown (dashed line). All solutions were dialyzed against distilled water before addition of 0.1 M KOH to pH 10.1. Protein concentration was 0.20 mg per ml; 0.10 M NaCl was present.
Fig. 8. Fluorescence of albumin-bound ANS as a function of pH. Various amounts of KOH or HCl were added to separate aliquots of Armour Fraction V BSA (Lot M 12005), which were then diluted to 7.0 mg per ml (10⁻⁴ M). The pH was recorded after addition of the fluorescent dye ANS (final concentration, 5 × 10⁻⁶ M) to each aliquot. The points indicate the fluorescence excited at 378 nm and observed at 460 nm for solutions of charcoal-treated BSA (Curve B), untreated BSA (Curve A), and treated BSA to which were added 3.0 eq of palmitate (Curve C). The temperature was 23°.

Fig. 9. Fluorescence spectra of ANS bound to BSA. The curves shown were obtained with the solutions of charcoal-treated BSA used in the experiment of Fig. 8, Curve A, at pH 5.0 (●—●), and at pH 3.28 (●—●). Excitation wave length was 378 μm; the spectra are corrected for detector response.

Fig. 10. Fluorescence spectra of human serum albumin preparations. Curve 1, crystalline HSA (Pentex, Lot 9); Curve 2, HSA crystallized four times (Nutritional Biochemicals, Lot 6941); Curve 3, HSA crystallized four times (Nutritional Biochemicals, Lot 7741). All three samples were in distilled water and were diluted to give an optical density of 0.657 cm⁻¹ at 280 μm; the optical densities were also equal at 272 μm, the wave length of excitation. The temperature was 23.0°, pH 2.9. Curve 1 is also the spectrum obtained from the three samples after they were treated with charcoal (see text).

for the presence of the unfolded or "F" form of serum albumin was at pH 3.33. The fluorescence emission spectrum of albumin-bound ANS at the pH 3.35 peak is significantly shifted toward the blue with respect to that at neutral pH, as shown in Fig. 9. The peak of the emission band at low pH is at 465 μm, as compared to 470 μm at neutral pH. This finding correlates well with the evidence presented by Stryer (31) that ANS fluorescence is shifted toward the blue when the fluorescent group is exposed to a hydrophobic environment. Presumably, the ANS molecule, which is already bound in a region of low dielectric constant at neutral pH, comes into contact with an even more hydrophobic area at pH 3.35. The peak in ANS fluorescence at pH 3.35 and the spectral changes shown in Fig. 9 occurred with both the treated and untreated samples used in the experiments of Fig. 8.

Effect of Charcoal Treatment on Acid-induced Expansion of Serum Albumin—Studies by Sogami and Foster (32) and by Foster et al. (33) have shown that albumin preparations are heterogeneous and that subfractions can be isolated which undergo acid-induced molecular unfolding at slightly different pH values. Such experiments were done with both charcoal-treated and untreated crystalline samples of BSA (Pentex, Lot Bx4) and HSA (Pentex, Lot 9), in order to see if charcoal treatment altered the characteritic pH of the N-F transition. No difference was found; the midpoints of the transitions were at pH 4.0 and 4.1 for the HSA and BSA samples, respectively, in 0.1 M NaCl, and were not changed by charcoal treatment.

Additional Observation—The fluorescence emission spectra of several different crystalline HSA samples were examined, and, surprisingly, they were found to be nonidentical. Fig. 10 shows the native protein spectra which are due to tyrosine and to the lone tryptophan (35). Two samples (Curves 2 and 3) show considerably less tryptophan emission than the HSA from Pentex. After treatment of these protein samples at pH 7 with charcoal (0.2 mg per mg of protein), all the spectra reverted to the same shape as in Curve 1, which itself did not change. Under these
conditions of low charcoal concentration and neutral pH, the FFA content was not affected, so that the relative quenching of the tryptophan emission at about 340 nm (Curves 2 and 3 of Fig. 10) may have been due to an impurity (not FFA) removable by charcoal. The presence of such impurities may explain the variations noted by Steiner and Edelhoch (34) in the fluorescence of HSA samples. These findings also suggest that the emission spectrum exhibited by the Pentex sample of HSA (Fig. 10, Curve 1) is the true fluorescence spectrum of HSA when excited at 272 nm. The spectrum of Curve 1 was also obtained from all three samples after they had been subjected to charcoal treatment at pH 3.0, which resulted in removal of FFA. The fact that charcoal treatment did not change the spectrum is a further indication that gross conformational changes are not induced, since the emission spectrum of HSA appears to be quite sensitive to such changes (35).

**DISCUSSION**

Since serum albumins are of such general importance, the present results, which show that acid-charcoal treatment is an effective defatting procedure, may be of some interest. Methods which have used treatment of albumin with ethanol or methanol to remove lipid impurities have been noted to result in a significant degree of protein aggregation (2, 4). Exposure of albumin to isooctane in Goodman's procedure (10) would seem to entail risks of similar denaturation effects, although this has not been documented. McMenamy (7) attempted to ascertain whether radioactive isooctane was removed in the final steps of Goodman's procedure, but the results were inconclusive. The presence of hydrocarbon might cause some objectionable conformational changes; indeed, Alfsen (36) has reported that n-heptane alters the optical rotation of BSA solutions.

Attention was focused mainly on the FFA content of serum albumin in this study, but it seems clear from the protein emission spectra (Fig. 10) that charcoal probably removes other contaminants as well. If albumin samples contain dinonyl ketone, as postulated by Bro and Sturtevant (3), this impurity would also be expected to be adsorbed by carbon. It should be noted that the method of acid-charcoal treatment described in the present work requires about 2 hours, including 1 hour of contact with charcoal and centrifugation time. No other method described for removing FFA from albumin is as rapid.

From the evidence presented in this paper, it seems that charcoal treatment is a gentle procedure which does not cause denaturation of serum albumin. This conclusion is supported by (a) absence of induced dimerization detectable by ultracentrifugation, (b) lack of rotatory dispersion changes, (c) retention of the ability of charcoal-treated BSA to form complexes with FFA, (d) lack of change in the pH of the N-F transition as determined by fluorescence emission, (e) ability of treated BSA to enhance the fluorescence quantum yield of ANS to the same degree as untreated BSA, (f) lack of effect on the emission spectrum of HSA, except for that attributable to the removal of impurities, and (g) similarity of the emission spectra of ANS bound to treated and untreated BSA at both pH 3.4 and pH 7.0. It is probably unnecessary to emphasize that it might still be possible to show some effect of charcoal treatment on the protein if other physical techniques were used. However, should such differences be found, one would be faced with the problem of deciding whether the treated or the untreated albumin were more "native."

It may at first seem somewhat surprising that acid-charcoal treatment has so little effect on the structure of serum albumin, since this protein undergoes a molecular unfolding at acid pH which exposes hydrophobic residues to the solvent (30), and charcoal has tremendous affinity for such hydrophobic surfaces. A possible explanation may be that at acid pH charcoal particles do in fact become tightly "coated" with albumin which is not subsequently released, but the bulk of the protein is then unable to reach the adsorbing surface within the micropores of the carbon. Lau et al. (37) have shown that albumin-treated Norit has different adsorptive properties than does untreated charcoal. They found that the albumin-treated Norit, which they call coated charcoal, would not adsorb protein-bound vitamin B12, but would adsorb B12 which was free in solution; but that both bound and free vitamin B12 were adsorbed by untreated charcoal. In the present study, it is likely that the charcoal particles are all coated with albumin, which then acts as a membrane permeable to FFA and other small molecules but not to other albumin molecules. Such a mechanism would help to explain why there was little or no change in protein structure, since those protein molecules remaining in solution would not actually come into contact with carbon. Thus, the tremendous surface area afforded by the micropores of activated charcoal (20) would still be available to small molecules such as FFA, but would be inaccessible to protein.

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