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A COMPARATIVE STUDY OF SOME PHYSICO-CHEMICAL PROPERTIES OF HUMAN SERUM ALBUMIN SAMPLES FROM DIFFERENT SOURCES—I

SOME PHYSICO-CHEMICAL PROPERTIES OF ISOIONIC HUMAN SERUM ALBUMIN SOLUTIONS

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Abstract—Human serum albumin samples from different sources were investigated. The fatty acid content of the albumin before and after deionization on a mixed bed ion-exchange column varied from sample to sample. When an albumin sample from one source was deionized under standard conditions the amount of fatty acid bound by the albumin was reduced to a reproducible amount. In samples from different sources, however, the amount bound varied considerably. Also the isoionic pH of the albumin varied from sample to sample. This variation could be attributed to the difference in the fatty acid content and the different number of titratable histidines and acid amino-acid residues in the albumin from different sources. It can be concluded from the specific conductance of these isoionic solutions that ions other than H^+, OH^- and protein are effectively removed by a mixed bed ion-exchange column. The specific conductance of the albumin samples is directly related to the isoionic pH. Therefore, the isoionic pH and the specific conductance of the albumins reflect the heterogeneity of the albumin samples with respect to their primary structure and fatty acid content.

Albumin† is an important carrier of endogenous and exogenous substances in blood [1–3]. Therefore, the binding of both non-physiological (i.e. drugs) and physiological substances are studied extensively. Not only organic compounds such as bilirubin and fatty acids but also small inorganic ions such as chloride and calcium are known to influence the binding of various substances to albumin [4–8]. Recently it has been observed that these small inorganic ions can affect the conformational change of albumin around neutral pH, the so-called N–B transition [7]. Therefore, it is advisable that ionic substances be removed from albumin samples before studies on the conformation or binding properties of albumin are carried out. An obvious way of doing this is to use a mixed bed ion-exchanger [6, 7]. One expects small inorganic ions to be effectively removed from an albumin solution by this method. Whether organic ions or non-ionic substances are removed by ion-exchange resins will depend on the one hand the strength of the protein binding of the substances and on the other hand on the affinity of these substances for the resin.

Already in 1970, Scheider and Fuller [9] reported a method for defatting albumin in the pH range 3.0–4.0 using buffered ion-exchange or surface adsorber resin columns. However, when the pH of an albumin solution is decreased below 4.0, the albumin passes the N–F transition and there is concomitant unfolding of the protein [10, 11]. Although some studies have shown that albumin, after being unfolded at pH below 4.0, folds back when the pH is increased to neutral pH, it is not known whether the albumin will still be native after it has been unfolded [12].

Therefore, we investigated the effectiveness of some ion-exchange and one surface adsorber resin in removing inorganic ions and fatty acids from albumin solutions when the system was not buffered. Furthermore, we compared some characteristics such as the pH and the specific conductance of some albumin solutions of various deionized albumin samples.

MATERIALS AND METHODS

The origin and lot number of the albumin samples used in this study are given in Table 1. Two albumin samples were prepared using a modification of the method of Hao [13, 14]. Concentrations of the albumin solutions were determined after deionization by drying at 105° in air until constant weight was achieved. The molecular weight of albumin was taken as 66,500. Unless otherwise stated, the albumin concentration used in the experiments varied between 3.5 and 3.7% (w/w). The ion-exchange resins Amberlite IR-120 (lot number 6259350) and Amberlite IRA-400 (lot number 2431220) and the

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† Albumin in this paper means human serum albumin, unless otherwise stated.

Table 1. Molar ratio of fatty acid to albumin of the various albumin preparations before and after the deionizing process*

Origin of the albumin samples	Code	r_{FA} (before)	n	r_{FA} (after)	n
Miles Laboratories Ltd., Stoke Court, Stoke Poges, Slough, England; fraction V, fatty acid free, lot 15	01	0.05 ± 0.01	5	0.04 ± 0.03	5
Miles Laboratories Ltd., Stoke Court, Stoke Poges, Slough, England; fraction V, lot 145	02	1.94 ± 0.04	8	1.07 ± 0.03	3
Behringwerke AG., Marburg, West Germany; lyophilized, purified, lot 455009-A	03	0.32 ± 0.01	5	0.20 ± 0.04	5
Sigma Chemical Company, St. Louis, Missouri, U.S.A.; crystallized, lyophilized, lot 108C-8085	04	1.04 ± 0.05	7	0.65 ± 0.08	8
Sigma Chemical Company, St. Louis, Missouri, U.S.A.; fraction V, lot 47C-04423	05	2.12 ± 0.08	6	2.05 ± 0.04	5
Preparation according to Hao modified by us, lot 810116	06	0.94	1	0.77	1
Preparation according to Hao modified by us, lot 810123	07	0.83	1	0.73	1

* r_{FA} denotes the molar ratio fatty acid to albumin.
Standard deviations and number of measurements (n) are given.

surface adsorber resin Amberlite XAD-2 were obtained from BDH Chemicals Ltd., Poole, England. The anion-exchange resin Amberlite IRA-400 was converted to the hydroxide form according to Salmon and Hale [15]. Before the ion-exchange resins were used they were washed four times with monodistilled water. The ion-exchange capacity of the anionic and cationic resin were 0.42 and 1.56 meq/ml, respectively, as measured according to Salmon and Hale [15]. The mixed bed ion-exchange column consisted of one volume Amberlite IR-120 to two volumes Amberlite IRA-400. The Amberlite XAD-2 resin was used without further purification. All other chemicals were of analytical grade (Merck, Darmstadt, West Germany or J. T. Baker, Deventer, The Netherlands).

So that the deionizing process could be studied, 25 ml of an albumin solution was pumped through the column. The procedure was monitored by measuring the conductance of the protein solution with a flow-through cell (Radiometer CDC 114) connected to a conductometer (Consort K 620). The process was continued for at least half an hour until constant reading was obtained. Unless otherwise stated, the bed-volume of the column was 2.2 ml. After this procedure the pH of the protein solution was measured with a combined glass electrode (Radiometer GK 2401 C) coupled to a pH meter (Radiometer PHM 62).

The molar ratio of fatty acid to albumin was measured by the method described by Chen [16], but we used a thymol blue solution [17] as titration mixture instead of the Nile blue A reagent.

The acid–base titrations of the albumin samples were carried out as described by Janssen and Wilgenburg [18]. These titrations were done at an ionic strength of 0.15 (KCl) and a protein concentration of about 1.3% (w/w).

All experiments were carried out at 25°.

Table 2. Removal of fatty acids from albumin sample 02*

Resin	r_{FA}^{02} 2.2 ml	4.6 ml
XAD-2	1.60	
IR-120	1.98	
IRA-400	0.89	0.74
IR-120 : IRA-400 = 1 : 2	1.07	0.82

* The first column shows the resins which were used. Also the bed-volume was varied (2.2 or 4.6 ml). The second and third columns give the ratios of fatty acid to albumin of sample 02 after the deionizing procedure. The r_{FA} of albumin sample 02 before deionizing was 1.94. The standard deviation in r_{FA} is about 0.05.

RESULTS AND DISCUSSION

Comparison of resins with respect to their effectiveness in the defatting procedure

Table 2 shows the molar ratio of fatty acid to protein (r_{FA}) of albumin sample 02 after it has been passed through columns with various resins. The initial r_{FA} of this sample was 1.94. As can be seen from Table 2, the anion-exchange resin and the mixed bed ion-exchange resin are most effective in defatting the albumin sample, whereas the cation-exchange resin does not defat the albumin at all. It was found that the defatting effectiveness increases with the volume of anion-exchanger. Since the number of functional groups of the anion-exchange resin exceeds by far the number of free fatty acid molecules in the albumin solution, this increase in effectiveness is probably due to an adsorption mechanism. However, since the cation-exchanger, unlike the anion-exchanger, does not defat the albumin, although the type of resin matrix is the same as for the anion-exchanger, ion-exchanging must also play a part in the defatting procedure. A possible explanation is

that the albumin molecule is adsorbed to the resin matrix before it can exchange its bound fatty acid anions with the resin. Continuing the procedure after reaching a constant conductance did not result in further defatting.

The surface adsorber resin XAD-2 defats the albumin sample 02 much less than the anion-exchanger (Table 2). Scheider and Fuller [9] observed better defatting of albumin with anion-exchange resins or surface adsorber resins than we did. This is due to the difference in the pH of the albumin solution used in the two procedures. Scheider used a buffered column in the pH range 3.0 to 4.0, whereas in our procedure no buffered system was used. If the mixed bed ion-exchange column is used, the pH varies between 7.3 and 4.7. At a pH below 4.0 the albumin probably releases its bound fatty acid molecules more easily because the albumin is unfolded. As already mentioned in the introduction it is not known whether the albumin will return to its native structure after it has been unfolded. Therefore, we did not expose the albumin samples to pH values lower than 4.0.

The effect of the deionizing process on the fatty acid content of various albumin samples, when the mixed bed ion-exchange resin is used, is summarized in Table 1. It can be seen from Table 1 that the fatty acid content of the albumin before deionizing varies from sample to sample. After the deionizing process the fatty acid content has been reduced significantly, but is still not the same for each sample. The amount of fatty acid removed from the albumin samples by the deionizing process is not the same either. However, the deionizing process yields a reproducible fatty acid content for each albumin sample, as can be seen from the standard deviations.

The pH and specific conductance of isoionic albumin solutions

When the albumin samples have been deionized, the albumin solutions are called isoionic, which means that the protein solution contains, besides dissolved protein, only the ions arising from dissociation of the solvent [19]. Such an isoionic albumin solution has a certain specific conductance and a pH, called the isoionic pH (pH_{iso}). Figure 1 shows the specific conductance of the isoionic solutions of some albumin samples as a function of the molar concentration protons at pH_{iso}. The following well-known expression for the specific conductance (κ) of a solution of albumin can be used

$$\kappa = \Sigma_i c_i \lambda_i / 1000, \quad (1)$$

where λ_i is the equivalent conductance of the ion i, and c_i its molar concentration [20]. Making the reasonable assumption that after deionizing only protons, hydroxylions and albumin are present, we can write

$$\kappa = (\lambda_{H^+}[H^+] + \lambda_{OH^-}[OH^-] + \lambda_p[P])/1000, \quad (2)$$

where λ_p stands for the equivalent ionic conductance of the albumin at pH_{iso} (Scm^2/mole) and [P] is the molar concentration of albumin.

From the literature [20] it is known that the values of λ_{H^+} and λ_{OH^-} are 350 and 198 Scm^2/mole, respectively. Since all pH_{iso} values of the various samples are near 5.0, $\lambda_{OH^-}[OH^-]$ can be neglected, whereas $\lambda_{H^+}[H^+]$ cannot. Therefore, Eqn. (2) becomes

$$\kappa = 0.35[H^+] + \lambda_p[P]/1000. \quad (3)$$

On the other hand, the best fit of the curve in Fig. 1, if the least-squares method for linear regression is used, is described by

$$\kappa = 0.37\,(\pm 0.03)\,[H^+] + (2.49\,(\pm 0.30))\,10^{-6} \quad (r = 0.97). \quad (4)$$

It should be noted here that Eqn. (3) holds for one albumin sample, whereas Eqn. (4) describes the behaviour of six different albumin samples. Apparently the contribution of the albumin to the measured conductance of the isoionic solutions can be considered to be more or less constant. The value of λ_p which follows from Eqn. (4) is 4.6 Scm^2/mole. Bruin and Os [21] found a value of 3.0 Scm^2/mole for the equivalent ionic conductance of a 3.6% (w/w) bovine serum albumin solution.

Another important conclusion to be drawn from these results is that ions other than H^+, OH^- and protein are effectively removed by the mixed bed ion exchange resin. The starting value of the specific conductance of the albumin solutions varied between 70 and 800 μScm^{-1}.

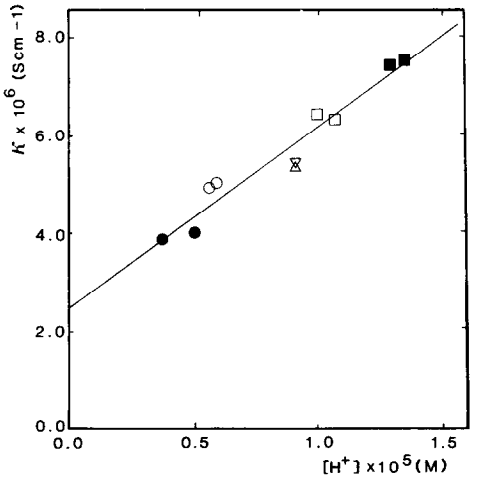

Fig. 1. Relationship between the specific conductance (κ) and the molar concentration protons of various isoionic albumin solutions. (●) = albumin 01; (○) = albumin 03; (■) = albumin 05; (□) = albumin 04; (▽) = albumin 06; (△) = albumin 07.

Acid–base titration curves of the albumin samples

The problem that still remains to be solved is why the samples display such a difference in isoionic pH. If the overall structure of the albumin samples is the same, there should be a difference in the ionic species firmly bound by the albumin. This difference can be roughly approximated as follows. Around pH_{iso} a change of one charge unit on the albumin molecule (Z_H) results in a change of about 0.1 in the pH [22]. This implies that there must be a difference of 4 to 5 charge units in albumin samples 01 and 05. Since the difference in r_{FA} is about two this cannot be fully explained by a difference in the number of bound

Table 3. Data obtained from the differential acid–base titration curves of the various albumin samples*

Code of the albumin sample	Z_I	Z_{II}	$Z_I - Z_{II}$	$Z_{II} - Z_{II}^{01}$	r_{FA}
Albumin 01	−5.1	−18.3	13.2		0.04
Albumin 03	−5.5	−18.8	13.2	−0.5	0.20
Albumin 04	−7.1	−19.3	12.2	−1.0	0.65
Albumin 05	−9.2	−21.6	12.4	−3.3	2.05

* Z_I and Z_{II} stand for the proton charge of the albumin at the first and the second maximum in the $-\Delta pH/\Delta Z_H$ vs $-Z_H$ curve, respectively. The last column gives the molar ratio of fatty acid to protein (r_{FA}) of the various albumin samples.

fatty acids. To elucidate this discrepancy the acid–base titration curves of the various albumin samples were measured. The data obtained from the differential titration curves of the various albumin samples, where $-\Delta pH/\Delta Z_H$ is plotted vs $-Z_H$, are given in Table 3. The first maximum in the differential titration curve (Z_I) is, when pronounced, by approximation, the maximum positive protonic charge of the protein (Z_{max}) minus the number of titratable carboxyl groups (N_{COOH}) [23, 24].

$$Z_I = Z_{max} - N_{COOH}. \quad (5)$$

The second maximum (Z_{II}) is Z_{max} minus N_{COOH} and the number of titratable histidines (N_{HIS}) and the terminal α-NH_2 group

$$Z_{II} = Z_{max} - N_{HIS} - N_{COOH} - 1. \quad (6)$$

Combining Eqns. (5) and (6) we can write

$$Z_I - Z_{II} = N_{HIS} + 1. \quad (7)$$

Since Z_{max} is equal to the sum of the number of titratable lysines (N_{LYS}), arginines (N_{ARG}), histidines (N_{HIS}) and the terminal α-NH_2 group, Eqn. (6) becomes

$$Z_{II} = N_{LYS} + N_{ARG} - N_{COOH}. \quad (8)$$

From Table 3, column 4, it can be seen that the number of titratable imidazole groups is not equal for all the albumin samples. The exact number of titratable histidines can be calculated from the values in column 4 of table 3 [23]. The calculated number of titratable histidines varies from 12.3 to 13.3 for the various albumin samples. According to the reported animo-acid composition [25, 26], human serum albumin should contain 16 histidines. Therefore, it can be concluded that not all histidines are titratable. Probably some of the proton binding sites of the histidines are masked by other groups in the protein or by unknown ligands. This may also explain why there is a difference in the number of titratable histidines in the various albumin samples. However, this is not the only difference in the samples. The results in colum 3 of Table 3 show that Z_{II} is not the same in the albumins from different sources. From Eqn. (8) it can be seen that this might be due to a variation in the number of titratable carboxyl groups. Column 5 in Table 3 gives the Z_{II} of the various samples, from which Z_{II} of sample 01, which contains no fatty acid, is subtracted ($Z_{II} - Z_{II}^{01}$). When $Z_{II} - Z_{II}^{01}$ is compared to the r_{FA}, it can be seen that the difference in fatty acid content is not solely responsible for the differences in the Z_{II} of the various albumin samples. Eqn. (8) shows that either the number of lysines and arginines has to decrease or the number of carboxyl groups has to increase. From the preparative procedures employed, it is known that adventitious alterations in the albumin can arise. For example loss of amide ammonia from some asparagine or glutamine residues results in extra aspartic acid or glutamic acid residues, which causes the formation of extra carboxyl groups [27, 28]. Probably these changes in the amino acids are the reason for the differences in the Z_{II} of the various samples, when Z_{II} is corrected for the influence of the fatty acids.

From the data in Table 3 and the number of lysines and arginines and from assumptions about the difference in pK_a between the carboxyl groups and the histidines, the isoionic pH of the albumin solutions can be calculated from the following equations [23, 24]

$$Z_H = Z_{max} - \Sigma_i \alpha_i N_i \quad (9)$$

where N_i is the number of groups in class i and α_i is the fraction of dissociated groups of class i, and

$$pH = pK_i + \log(\alpha_i/1 - \alpha_i) - 0.868\, wZ_H \quad (10)$$

where pK_i is the intrinsic pK_a value of the groups i, and w is the electrostatic interaction factor. In Table 4 the calculated isoionic pH (pH_{iso}(calc)) and the measured pH_{iso} of the albumin samples are given. It is assumed that the difference between the pK_a of the carboxyl groups and the histidines is 2.8 [23] and that the number of lysines and arginines is 81 [26].

As can be seen from Table 4, pH_{iso} and pH_{iso}(calc) do not differ by more than 0.1 unit. This means that the numbers of groups of titratable amino-acid residues, determined from the titration curves and the assumptions mentioned above, are very reasonable.

Table 4. The measured and calculated isoionic pH of some albumin samples*

Code of the albumin sample	pH_{iso}	pH_{iso}(calc)
Albumin 01	5.29	5.24
Albumin 03	5.23	5.21
Albumin 04	5.00	5.10
Albumin 05	4.89	4.99

* pH_{iso} and pH_{iso}(calc) denote the measured and calculated isoionic pH of the albumin samples, respectively.

From this paper it can be seen that after albumin has been deionized a reproducible amount of fatty acid remains bound to the albumin. It is not possible to reduce the fatty acid content to zero by deionizing. However, since effective defatting procedures are carried out at a pH below 4.0 [9, 16] it may be preferable to work with albumin that contains some bound fatty acid. Furthermore it is known that fatty acids enhance the stability of the albumin molecule [1]. The fatty acid content is partially reflected in the acid–base titration curves of the various albumin samples. Both the fatty acid content and the heterogeneity of the samples with respect to the primary structure have to be taken into account to explain the differences between the titration curves of the various albumin samples. From calculations it is seen that these factors also explain the differences in the pH_{iso} of the albumins. Therefore, the pH_{iso}, or the specific conductance, which is directly related to the pH_{iso}, reflects the heterogeneity of the albumin samples with respect to their structure and fatty acid content.

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