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HETEROGENEITY OF RAT PLASMA ALBUMIN AND DRUG BINDING

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Abstract—Rat plasma albumin fractionated by Sephadex G-75 (superfine) gel filtration from freshly prepared rat plasma was further separated into three major fractions by chromatofocusing column chromatography. All the fractionated albumins had high binding affinity for the fluorescent probe 8-anilino-1-naphthalenesulfonic acid, as the model compound for acidic drugs, and were found to be immunologically identical.

Plasma albumin is well known as a carrier protein for the transport of endogenous and exogenous substances. Among the various important functions of plasma albumin, that of binding with many kinds of drugs, particularly acidic drugs, is important from a biopharmaceutical standpoint. Numerous experiments on the binding capacity of albumin with drugs have been carried out *in vitro* for the clarification of the interactions of drugs with albumin *in vivo* [1–3]. Aggregated albumin has been found to be present in plasma albumin *in vitro* [4]. Stored albumin samples such as those commercially available contain aggregated albumin. It has not been observed, however, in fresh blood.

The presence of mercapt and non-mercapt albumins in plasma albumin has been reported as one of the reasons why albumin is heterogeneous [5]. This heterogeneous albumin has generally been used in the study of the binding of drugs to albumin. However, from a biopharmacuetical standpoint, elucidation of the effects of albumin heterogeneity on drug binding is very important. Consequently, the present research was conducted to clarify this matter, using freshly fractionated rat plasma albumin.

MATERIALS AND METHODS

Materials. 8-Anilino-1-naphthalenesulfonic acid sodium salt (ANS†), Coomassie Brilliant Blue-R 250, bovine serum albumin (fraction V), ovalbumin (grade VI), myoglobin (type III) and cytochrome *c* (type VI) were purchased from the Sigma Chemical Co. (St Louis, MO, U.S.A.). Heparin was purchased from the Novo Industries Co. (Denmark). Sephadex G-75 (superfine), Sephadex G-75, Sephadex G-25 (fine), PBE 94 and Polybuffer 74 were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). A centriflo ultrafiltration membrane cone CF 25 was purchased from the Amicon Co. (Danvers, MA, U.S.A.). Agar Noble was purchased from Difco Laboratories (Detroit, MI, U.S.A.). Sodium azide was purchased from Nakarai Chemicals, Ltd. (Kyoto, Japan). NEFA-Test Wako was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). All other chemicals were of analytical grade.

Blood sample collection. Male Wistar rats (225–275 g body weight) under light ether anesthesia had their femoral vein injected with heparin (100 units/100 g body weight). Blood samples were taken from a cannula in the carotid artery. Immediately following this, the blood was centrifuged for 10 min at 3000 rpm to obtain the plasma.

Fractionation of plasma albumins. Plasma (10–15 mL) was applied to a Sephadex G-75 (superfine) column (6 × 85 cm) previously equilibrated with 0.05 M Tris–HCl buffer (pH 7.4) and eluted with the same buffer at a rate of 10 mL/hr. Samples (3 mL) of the effluent were collected by means of a fraction collecter FRAC-100 (Pharmacia Fine Chemicals), and the protein concentration in the effluent was measured by absorbance at 280 nm, using a Pharmacia Single Path Monitor UV-1. The third of three major fractions was collected as a crude albumin fraction and concentrated by ultrafiltration using a Centriflo ultrafiltration membrane cone. This concentrated fraction of the crude albumin was separated further by chromatofocusing column chromatography. The constituents of the buffer containing this concentrated albumin fraction were replaced with 0.025 M imidazole–HCl buffer (pH 7.4) by ultrafiltration using a Centriflo ultrafiltration membrane cone. The albumin fraction thus obtained was applied to a PBE 94 column (1 × 35 cm) equilibrated with this buffer and eluted with the buffer consisting of the polybuffer diluted nine times with distilled and deionized water, adjusted to pH 4 with HCl, at a rate of 18–30 mL/hr; every 3 mL of the effluent was fractionated. The proteins of the eluate were detected by absorbance at 280 nm, using a Pharmacia Single Path Monitor UV-1. After completion of the separation, the column was washed with 1 M sodium chloride solution. The components of the polybuffer contained in the albumin fractions, after each albumin fraction was concentrated by ultra-

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† Abbreviations: ANS, 8-anilino-1-naphthalenesulfonic acid; PAGE, polyacrylamide gel electrophoresis; and SDS, sodium dodecyl sulfate.

filtration, were removed by eluting with deionized and distilled water through a Sephadex G-75 column, and then each fractionated albumin was lyophilized. Each lyophilized albumin fraction was then defatted with charcoal under pH 2.0–2.5 according to a slight modification of the method of Chen [6]. After the defatted albumins were eluted with water through a Sephadex G-25 (Fine) column, they were lyophilized again. All the procedures of plasma albumin fractionation were carried out at 4° throughout.

Fatty acid content of the albumins. The content of fatty acids bound to the albumins was determined using NEFA-Test Wako which is a slight modification of the method of Duncombe [7].

SDS–polyacrylamide gel electrophoresis. SDS–polyacrylamide gel electrophoresis (SDS–PAGE) (10%) of the fractionated albumins was carried out according to the method of Weber and Osborn [8]. The gel bands were stained with 0.25% Coomassie Brilliant Blue-R 250. The molecular weight of the fractionated albumins was determined by a Ferguson plot [9].

Preparation of antibody to fractionated albumins. Male New Zealand white rabbits were injected subcutaneously with a solution containing 2 mg of fractionated albumin in 0.15 M NaCl (500 μL) and complete Freund's adjuvant (500 μL) in at least five sites on the back. The injections were given once a week for 5 weeks. The presence of rabbit antiserum to the fractionated albumins was tested by double-immunodiffusion using agarose discs made from 1% agarose dissolved in 0.01 M phosphate-buffered saline, pH 7.2, containing 0.1% sodium azide [10].

ANS binding to plasma albumin. ANS binding to each fractionated albumin was carried out at room temperature in 0.25 M sucrose–0.05 M Tris–HCl buffer, pH 7.4, by the fluorescence titration method (excitation wavelength, 380 nm; emission wavelength, 480 nm) [11], using a Hitachi Fluorescence Spectrophotometer 650–10S. The protein concentration was adjusted to have an absorbance of 0.03 at 280 nm. The ANS binding data were fitted to the following equation (1) by a non-linear least squares computer program (MULTI program [12]).

$$C_b = \frac{n_1(P)C_f}{K_1 + C_f} + \frac{n_2(P)C_f}{K_2 + C_f} \qquad (1)$$

where C_b is the concentration of ANS bound to albumin, C_f is the unbound ANS concentration, P is the concentration of albumin, and K_i and n_i ($i = 1, 2$) are the dissociation constants and the number of binding sites at binding site i on the albumin molecule respectively.

Spectral measurements. The absorption and tryptophan fluorescence spectra of albumin were measured at room temperature and 25°, respectively, using a Shimazu UV-Visible Recording Spectrophotometer UV 260 and a Hitachi Fluorescence Spectrophotometer 650–60, respectively.

pH and thermal effects on plasma albumin. Tryptophan fluorescence spectra of albumin were measured at thermally controlled temperatures in 0.1 M KCl using a Hitachi Fluorescence Spectrophotometer 650–60. The corrected fluorescence emission spectra were obtained using rhodamine B as the quantum counter for fluorescence spectra. The pH of the solution containing rat plasma albumin in the cuvette was adjusted by adding a small aliquot of diluted HCl to the cuvette. The temperature of the sample in the cuvette was raised by continuously increasing that of the thermo bath. The temperature was determined with a digital surface thermometer HLC-60 Type E (Anritsu Meter Co., Ltd., Tokyo, Japan).

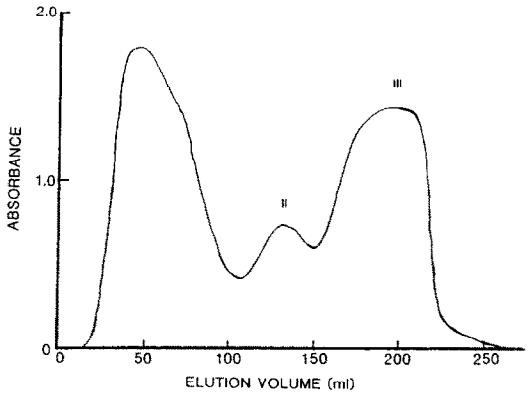

Fig. 1. Fractionation of rat plasma by Sephadex G-75 (superfine) gel filtration. Fractions consisting of 3 mL of effluent were collected. The detection of eluted protein was carried out by the absorbance at 280 nm with a UV monitor. The fractionation was denoted by the elution volume collected over the 40 hr from the start of the elution. The three major fractions eluted were denoted as fractions I, II and III.

RESULTS

Separation of rat plasma albumin. Rat plasma was separated by gel filtration using Sephadex G-75 (superfine) and three major fractions were obtained (Fig. 1), which agrees with the result reported by Flodin and Killander [13]. According to their classification, the albumin fraction was denoted as fraction III in Fig. 1. It was further purified by chromatofocusing (Fig. 2). The proteins eluted

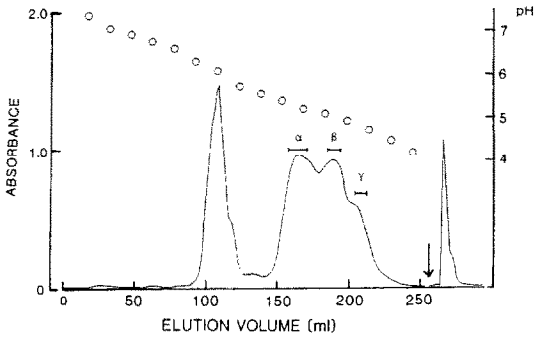

Fig. 2. Fractionation of rat plasma albumin (fraction III) by chromatofocusing. Fraction III in Fig. 1 was separated by chromatofocusing. Open circles indicate the pH values of the effluent. Each albumin fraction was denoted as α, β- and γ-albumins. The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

Table 1. Fatty acid contents of α-, β- and γ-albumins*

Preparations	Fatty acid (mol/mol protein)
α-Albumin	0.041 ± 0.027
β-Albumin	0.108 ± 0.047
γ-Albumin	0.149 ± 0.088

* α-, β- and γ-Albumins separated by chromatofocusing were defatted by the defatting procedure of Chen [6]. Values are means ± SE of three determinations; there was no statistically significant difference among them.

around pH 6 were slightly red in color. Major proteins were eluted around pH 5, and the three major fractions were designated as α-, β- and γ-albumins. Then these fractionated albumins were defatted by the defatting procedure of Chen [6], and the fatty acid contents of defatted α-, β- and γ-albumins were determined according to a slight modification of the method of Duncombe [7] (Table 1). There was no statistically significant difference among their fatty acid contents. Thus, characterization of defatted α-, β- and γ-albumins was carried out throughout this study, because fatty acids are well known to affect various functions of albumin such as ligand binding to the albumin [14].

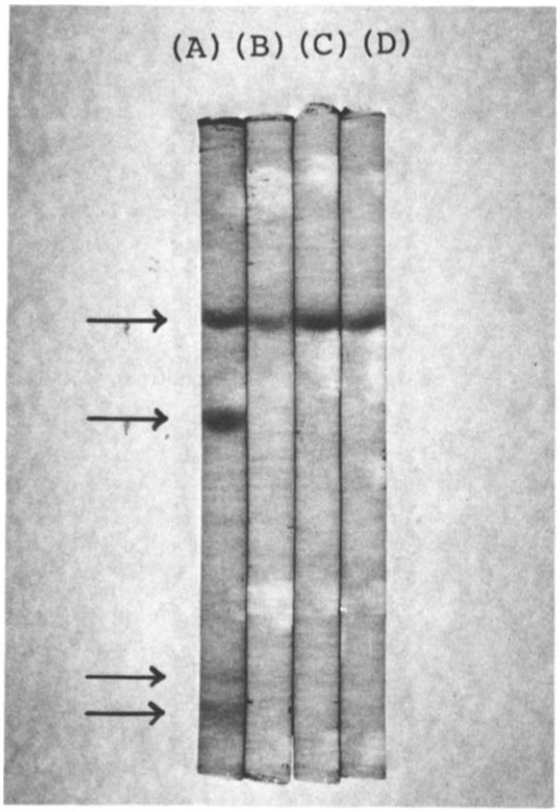

Fig. 3. SDS–polyacrylamide gel electropnoresis. The gel bands were stained with Coomassie Brilliant Blue. The lanes were loaded as follows: (A) molecular weight standards indicated by arrows, from top: bovine serum albumin, 67,500; ovalbumin, 45,500; myoglobin, 18,800; and cytochrome *c*, 12,400; (B) α-albumin; (C) β-albumin; and (D) γ-albumin.

SDS–PAGE of fractionated albumins. SDS–PAGE of α-, β- and γ-albumins demonstrated in all cases a single band stained with Coomassie Blue (Fig. 3). Their molecular weights, determined by Ferguson plots, were each about 67,000.

Immunological characteristics of fractionated albumins. Ouchterlony double-immunodiffusion of rabbit antiserum to the fractionated albumins showed clearly a single identical line of fusion between rabbit anti-albumin serum and α-, β- and γ-albumins (Fig. 4).

ANS binding to albumin. ANS, an anionic fluorescent probe, was used as the model compound for anionic drugs. The binding of ANS to the fractionated albumins was studied by the fluorescence titration method [11]. The results are shown in Fig. 5 by Rosenthal plots. The binding data of ANS to α-, β- and γ-albumins showed the best fit with Equation 1, assuming two kinds of binding sites. The binding parameters obtained are shown in Table 2.

Spectroscopic characteristics of albumin. The tryptophan fluorescence spectra of α-, β- and γ-albumins are shown in Fig. 6. The fluorescence intensity of γ-albumin was less than the intensity of the others. Moreover, the spectrum of γ-albumin differed slightly from those of the others, with a shoulder around 333 nm and a small red shift. The maximum fluorescence wavelength of γ-albumin was about 2 nm longer than those of the other two. All three albumins showed essentially the same absorption spectra, although in the case of β-albumin the absorbance ratio at 280 nm and 295 nm differed slightly from that of the other two (Data not shown).

The N–F transition of albumin was studied by tryptophan fluorescence according to the method of Sogami *et al.* [15]. Typical tryptophan fluorescence spectra of α-, β- and γ-albumins at several pH values of the medium are shown in Fig. 7 to present the spectral changes distinctly. The fluorescence spectra of α- and β-albumins shifted as a whole downward to lower wavelengths as the pH value decreased. The changes of intensities and peaks of the spectra of α- and β-albumin with pH are shown in Fig. 8, A and B. The peaks of α- and β-albumins were kept constant below pH 3 and above pH 3.9. However, the tryptophan fluorescence spectra of γ-albumin

Table 2. Binding parameters of ANS binding to α-, β- and γ-albumins*

	n_1	K_1 (nM)	n_2	K_2 (nM)
α-Albumin	0.85	93	1.1	734
β-Albumin	0.87	58	1.6	601
γ-Albumin	0.92	133	0.97	806

* The albumin concentration for all the albumins was adjusted to have an absorbance of 0.03 at 280 nm. Correlation coefficients of the binding results analyzed by Equation 1 were 0.9970, 0.9998 and 0.9997 for α-, β- and γ-albumins respectively. K_i and n_i ($i = 1, 2$) are the dissociation constants and the number of binding sites at binding site i on the albumin molecules.

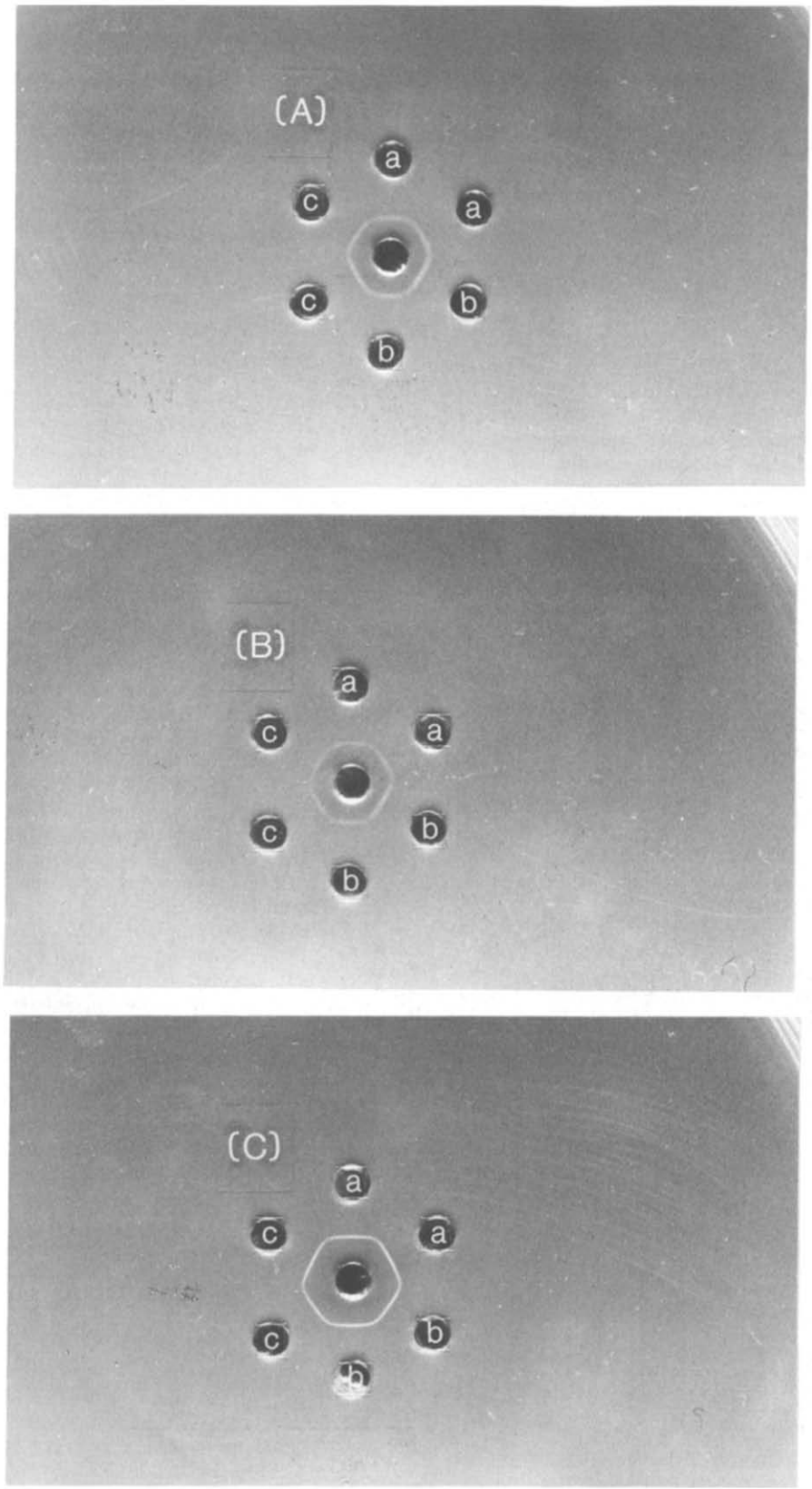

Fig. 4. Ouchterlony double-immunodiffusion on an agarose disc. Rabbit antiserum (5 μL undiluted) was in the center: (A) rabbit antiserum to α-albumin, (B) rabbit antiserum to β-albumin, and (C) rabbit antiserum to γ-albumin. α-, β- and γ-Albumins (5 μL of 1 mg/mL 0.01 M phosphate-buffered saline, pH 7.2) were in the peripheral wells labelled a, b and c respectively.

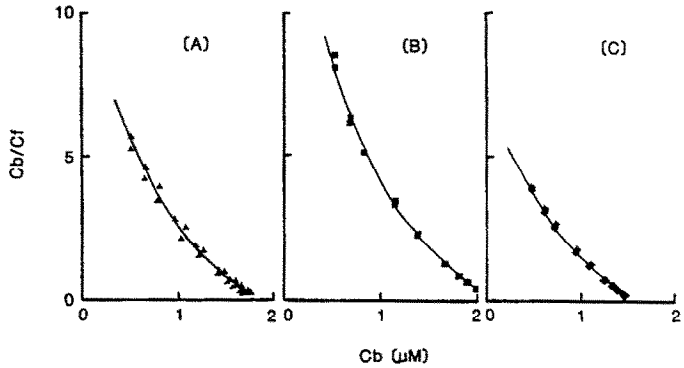

Fig. 5. Rosenthal plots of ANS binding to α-, β- and γ-albumins. ANS binding was studied with a 0.03 absorbance of the albumin solution at 280 nm. Closed symbols were the observed values. The lines indicate the calculated curves obtained from the values of the binding parameters in Table 2. Key: (A) α-albumin, (B) β-albumin, and (C) γ-albumin. C_b is the concentration of ANS bound to albumin, and C_f is the unbound ANS concentration.

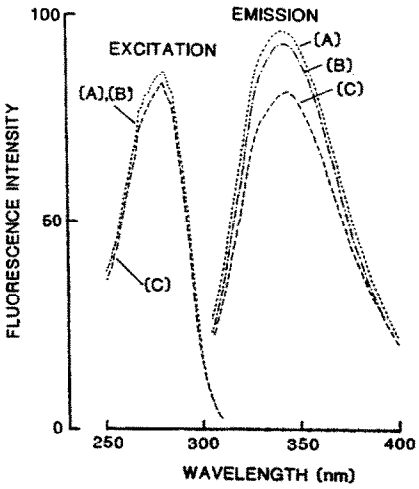

Fig. 6. Tryptophan fluorescence spectra of α-, β- and γ-albumins. Fluorescence emission spectra excited at 295 nm and excitation spectra emitted at 350 nm were measured at 25°. The albumin concentration was adjusted to 0.1 as the absorbance at 280 nm in deionized and distilled water. Key: (A) α-albumin, (B) β-albumin, and (C) γ-albumin. The excitation spectra of α- and β-albumins were similar.

showed a different change. As the pH value decreased, the peak above pH 3.9 decreased and finally became a shoulder below pH 3 but, in contrast, the shoulder above pH 3.9 increased and finally became a peak below pH 3. Typical tryptophan fluorescence spectra are shown in Fig. 7C. The changes of intensities and peaks of the spectra of γ-albumin with pH are shown in Fig. 8C. An examination was also made of thermal effects on the tryptophan fluorescence of albumin (Fig. 9). The tryptophan fluorescence intensity of each albumin decreased with temperature and the maximum peak of each albumin shifted to the lower wavelength at higher temperatures. In the case of γ-albumin, the fluorescence spectra showed two peaks over about 50°.

DISCUSSION

The present study reveals the heterogeneity of fresh rat plasma albumin and, thus, the effects of plasma albumin heterogeneity on drug binding to albumin were studied using purified rat plasma albumin. This albumin was separated by Sephadex G-75 (superfine) gel filtration and then fractionated newly

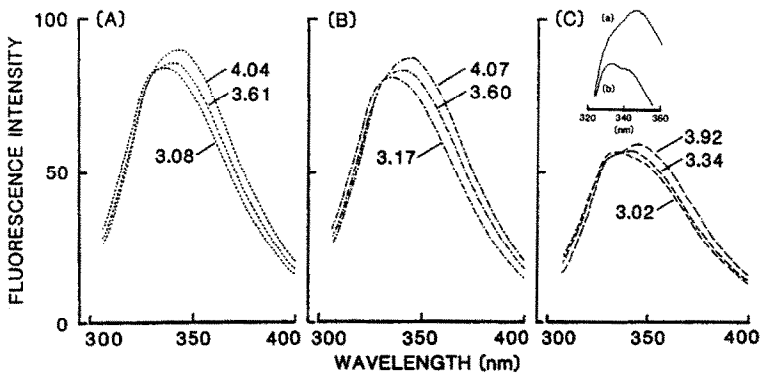

Fig. 7. Effect of pH on tryptophan fluorescence spectra of α-, β- and γ-albumins. The excitation wavelength was 295 nm. The albumin concentration was adjusted to 0.1 by the absorbance at 280 nm. Key: (A) α- albumin, (B) β-albumin, and (C) γ-albumin. The values in the figures indicate pH values of the media. Two typical fluorescence spectra of γ-albumin were inserted in panel C: (a) pH 4.41 and (b) pH 2.72, where the fluorescence spectra were not normalized in order to visualize them clearly.

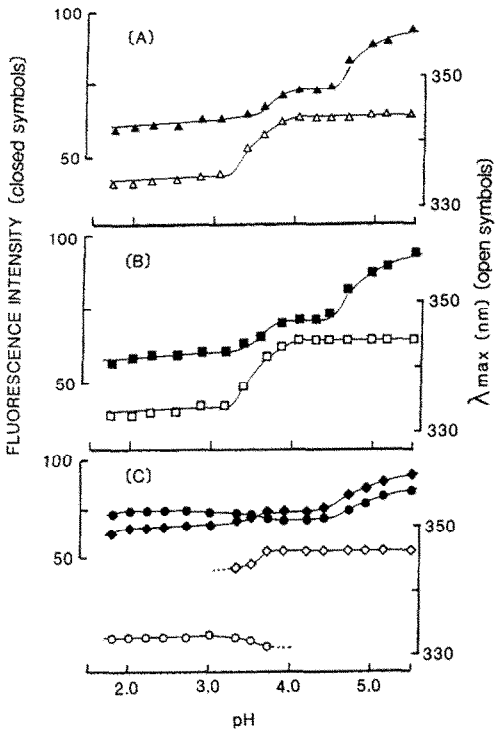

Fig. 8. Effect of pH on tryptophan fluorescence of α-, β- and γ-albumins. The data in Fig. 7 were used including data not presented in the figure. Key: (A) α-albumin, (B) β-albumin, and (C) γ-albumin. Fluorescence intensities at 350 nm, which were excited at 295 nm, were plotted against the pH of the medium: (▲) α-albumin, (■) β-albumin, and (◆) γ-albumin. Fluorescence intensities at 330 nm, which were excited at 295 nm, versus pH: (●) γ-albumin. The maximum fluorescence emission wavelengths (λ_{max}): (△) α-albumin, (□) β-albumin, (◇) λ_{max} of γ-albumin observed over approximate pH 3.9, and (○) λ_{max} of γ-albumin observed below approximate pH 3.0.

into three components by chromatofocusing column chromatography (Figs. 1 and 2). Plasma albumin is well known to bind tightly with acidic drugs. However, studies on drug binding with plasma albumin generally have been conducted with heterogeneous albumin. Thus, from biopharmaceutical and pharmacological standpoints, clarification of the manner in which these components contribute to the binding of drugs with albumin is very important. For this purpose, a fluorescent probe, ANS, was used as a model compound to study the binding of acidic drugs to the various components of albumin (Fig. 5 and Table 2). ANS bound tightly to α-, β- and γ-albumins, and its affinities for the albumins, although somewhat different, were similar. This suggests that three different albumins have strong and similar binding affinities toward acidic drugs and, therefore, that the binding characteristics of the heterogeneous albumin which have been studied so far can be used as a good estimate for each albumin fractionated in this study.

The separated components of plasma albumin were characterized by tryptophan spectroscopy. α- and β-Albumins were similar with respect to tryptophan fluorescence (Fig. 6). However, γ-albumin could be distinguished from the other two by the slight difference in their tryptophan. The same characteristics in these components as were observed in the spectra were noted with respect to pH and thermal effects (Figs. 7–9). The change in the tryptophan fluorescence of each albumin according to pH was due probably to the N–F transition as observed in human [16] and bovine [15] serum albumin. However, the N-F transition in rat albumin occurred at a lower pH, about 0.5 pH unit, than that in the case of human and bovine albumins. The maximum peak in the tryptophan fluorescence spectra of α- and β-albumins shifted to the lower wavelength with the decrease of pH. However, the tryptophan fluorescence spectra of γ-albumin showed a different pH-profile from the other two. As the pH value decreased, the peak of γ-albumin which was observed above pH 3.9 decreased and finally became a shoulder below pH 3, but the shoulder above pH 3.9 increased and finally became a peak below pH 3 (Fig. 7C). These differences in the properties of the tryptophan fluorescence in γ-albumin from those of α- and β-albumins may be due not only to the N-F transition but also to the heterogeneity of γ-albumin. This is possibly supported by the results of

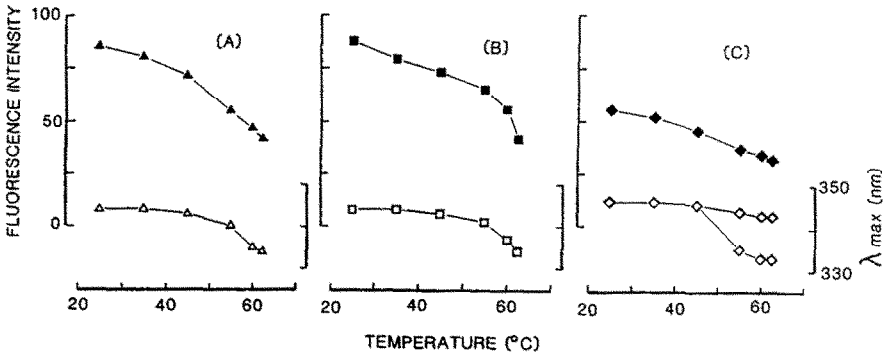

Fig. 9. Thermal effect on tryptophan fluorescence of α-, β- and γ-albumins. Fluorescence was measured as described in Fig. 7 and Materials and Methods. Key: (A) α-albumin, (B) β-albumin, and (C) γ-albumin. The data are shown in the same way as described in the legend of Fig. 8. The closed and open symbols indicate fluorescence intensity in arbitrary units and maximum fluorescence emission wavelength respectively. In the case of γ-albumin, the fluorescence emission spectra with two peaks appeared over about 50°. Thus, the wavelengths of these two peaks are shown at a higher temperature.

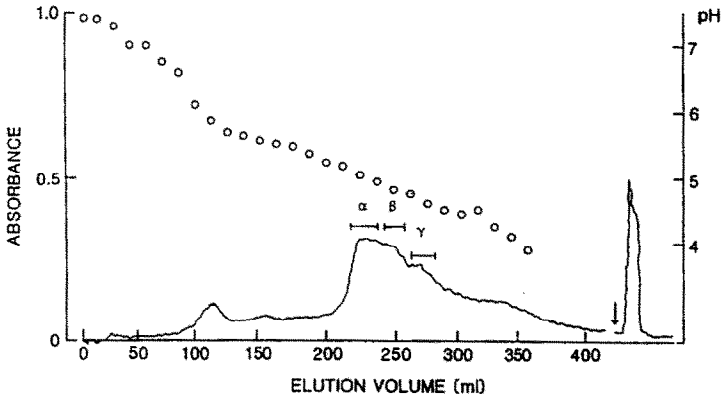

Fig. 10. Fractionation of defatted rat plasma albumin (fraction III) by chromatofocusing. The albumin (fraction III) in Fig. 1 was defatted by the defatting procedure of Chen [6], and the essentially defatted albumin was separated by chromatofocusing. Open circles indicate pH values of the effluent. The column was eluted with 1 M sodium chloride solution after the fraction indicated by an arrow.

experiments on thermal effects on albumin (Fig. 9). Meanwhile, each SDS–PAGE of the fractionated albumins showed a single band (Fig. 3). Ouchterlony double-immunodiffusion of rabbit anti-α-, β- and γ-albumin serum showed an immunological cross-reactivity between the antiserum and α-, β- and γ-albumins (Fig. 4). This indicates that the fractionated plasma albumins are immunologically identical. Thus, the fluorescence spectral change in the fractionated albumins with pH and thermal change likely resulted from change in the structure of albumin and, furthermore, the heterogeneity of γ-albumin, which was not detected immunologically and electrophoretically, may be responsible for the tryptophan fluorescence profiles associated with the pH and temperature changes.

The presence of endogeneous substances on albumin molecules might separate the plasma albumin (fraction III) into three different albumin fractions by chromatofocusing since the plasma albumin was defatted after fractionation. Fatty acids are well-known endogenous substances which generally bind to the plasma albumin. Thus, the effect of fatty acids bound to the albumin on the separation of the albumin by chromatofocusing was examined. The plasma albumin (fraction III) was defatted by the defatting procedure of Chen [6], before fractionation by chromatofocusing. This defatting procedure was repeated twice to remove fatty acids from the albumin as much as possible. Fatty acid contents of albumin (fraction III) and its defatted albumin were determined according to a slight modification of the method of Duncombe [7] (Table 3). The defatted albumin was completely free from fatty acids. The defatted albumin thus obtained was further separated by chromatofocusing (Fig. 10). The elution pattern was similar to that in Fig. 2, although the elution pattern was less distinct. It should be noted that three major fractions also appeared around pH 5 in the case of the defatted albumin. Each fractionated albumin in Fig. 10 also demonstrated the same spectroscopic characteristics as those of α, β- and γ-albumins shown in this study respectively. Consequently, it is clearly evident that the heterogeneity of plasma albumin does not result from the presence of fatty acids.

In conclusion, rat plasma albumin known for its tight binding with acidic drugs consists of at least three albumin components.

Table 3. Fatty acid content of plasma albumin and defatted albumin*

Preparations	Fatty acid (mol/mol protein)
Plasma albumin (fraction III)	6.845 ± 0.379
Defatted albumin	0.035 ± 0.013

* The plasma albumin (fraction III) was defatted twice by the defatting procedure of Chen [6]. Values are means ± SE of three determinations.

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