A pH Gradient Partition Chromatography of Plasma Albumin.

An Attempt to Separate into Subfractions*

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血清アルブミンの pH 勾配分配クロマトグラフィー

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INTRODUCTION

It has been pointed out that plasma albumin undergoes a transformation between N and F forms depending on pH at a pH region 3.0–5.0(2). Sogami and Foster(8) recently suggested that there might exist a large number of closely related albumin species having slightly different types of tertiary structures. Each species has its own characteristic pH at which the N–F transformation takes place, and the transformation is extremely sharp being accompanied by a 12 hydrogen transfer. Assuming a Gaussian type distribution of a population of these species, we can properly explain the observed pH dependence of the N–F transformation.

Because of the much lower water solubility of F form and its enhanced solubility in organic solvents (5), partition chromatography seems to be useful to separate albumin into two subfractions corresponding to N and F forms. Moreover, we can expect that many albumin species might be separated by a pH gradient elution because of their characteristic pH dependences.

Under these considerations, a pH gradient partition chromatography was attempted for plasma albumin.

EXPERIMENTAL

Materials — Crystalline bovine plasma albumin(BPA) was obtained from Pentex Inc.(Lot 9). Human mercaptalbumin(HMA) was prepared from three times recrystallized mercaptalbumin dimer by means of mixed bed ion exchange resin column recommended by Dintzis(4).

Silane–treated kieselguhr was prepared from Celite 545 obtained from Johns–Manville Co. Ltd., according to the method described by Porter(7).

Commercial diethylene glycol monobutyl ether(butyl carbitol) was fractionally distilled from sodium borohydride under a reduced pressure in an atmosphere of nitrogen. Only fractions having absorbances in a 1 cm cell less than 0.07 at 279 mμ were used.

Sucrose and other reagents used in this experiment were purest grade reagents commercially available and used without any purification procedure.

Phase System — Two liquid phases could be produced by mixing butyl carbitol, sucrose and aqueous buffer solution. The following was the composition of the phases used in this experiment : sucrose , 34 % ; buffer solution , 42 % ; butyl carbitol , 24 % (expressed using grams for sucrose and milliliters for the other two, for the sake of convenience). The volume of the upper phase(organic phase) was approx–

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imately half that of the lower phase(aqueous phase) at 24°C. The buffer solutions were composed of citric acid, trisodium citrate and potassium chloride. The concentartions of citrate and potassium chloride in the buffer solutions were 0.01 M and 0.2 M, respectively. Changing the pH of the buffer, the pH of the two phases could be changed without any change in the phase equilibrium.

Preparation of column and elution — To the silane-treated kieselguhr was added half its weight of the stationary phase(organic phase) having pH 2.8–3.1 and mixed well in a beaker with a spatula. Moving phase(aqueous phase), 2.5 times larger volume than that of the stationary phase mixed, was added to produce a smooth cream. Being free of lumps and air bubbles by stirring, it was poured gradually into a glass tube which has a sintered glass filter at bottom. This column was allowed to stand while draining of the excess of the moving phase had been achieved under gravity alone. More moving phase was poured and further drainage was continued until the washings showed absorbances at 279 mμ indicating no appreciable amounts of impurities.

One percent BPA solution, which was prepared by dissolving BPA in the moving phase and adjusting pH to 2.6–3.0 with 3 N hydrochloric acid, was poured on the top of the column. When it had almost sunk into the column, elution was started.

A pH gradient elution could be attained by mixing two moving phases having different pH, keeping the phase equilibrium unaltered. Moving phase of higher pH(~4.7) was introduced from a reservoir into an air-tight mixing vessel, which had only moving phase of lower pH(2.8–3.1) at the beginning of the elution. A pressure of approximately 1.3 m water head was applied by lifting the reservoir to obtain a sufficient flow rate during the elution.

Absorbances of effluents were measured at 279 mμ using a Beckmann DU Spectrophotometer after twofold dilution with deionized water, since effluents sometimes showed turbidity due to a phase disturbance.

Contact of the solvent with rubber and plastics should be avoided, since it always resulted in an extraction of many impurities which increased the absorbance at 279 mμ.

For the chromatography in which 100 mg albumin were used, 35 g silane-treated kieselguhr were packed into a tube of 2.3 cm diameter. In other cases, experimental conditions were made to be almost the same as in this case.

All experiments were carried out at 24±1°C.

RESULTS AND DISCUSSION

Preceding a chromatography, the partition coefficients of BPA were measured. To do this, the phase system containing BPA was allowed to stand overnight to complete the partition avoiding a vigorous shaking which might cause protein denaturation, after which absorbances of the both phases at 279 mμ were measured separately. These results are shown in Fig. 1. The partition coefficient is expressed as the ratio of concentration in organic phase to that in aqueous phase. A remarkable change in partition coefficient can be seen accompanying changing pH.

The chromatograph dealing with 100mg BPA is shown in Fig. 2. It can be seen that BPA is distributed among effluents whose pH is within a region of 3.4–4.0. This is a result which could be expected from the partition behavior. These fractions contained in tubes of No. 18–20, 21–23 and 24–27 were separately combined, dialized and lyophilized. The three fractions thus obtained are designated as BI–1, BII–1 and BIII–1, respectively.

Ultracentrifugal analyses were made for these fractions with a Spinco Model E Ultracentrifuge, which showed that all the fractions contained much dimer as shown in the Table. The starting BPA used in this experiment was that purified by the Dintzis ion exchange column(4). It also contained much dimer

according to ultracentrifugal analysis. Therefore, pure monomer* was isolated from Pentex Lot 9 by Sephadex G−200 column and used in the next experiment.

The chromatography scale was enlarged for 1 g BPA. The chromatograph obtained is qualitatively similar to that for 100 mg BPA. These fractions were combined into four fractions and designated as BI−2, BII−2, BIII−2 and BIV−2 in order of the pH at which they came out. These fractions also contained dimer as shown in the Table, although the dimer contents in this case are small compared with those in BI−1 series. It can be seen that the earlier fraction has more dimer than the later fraction has, in either case.

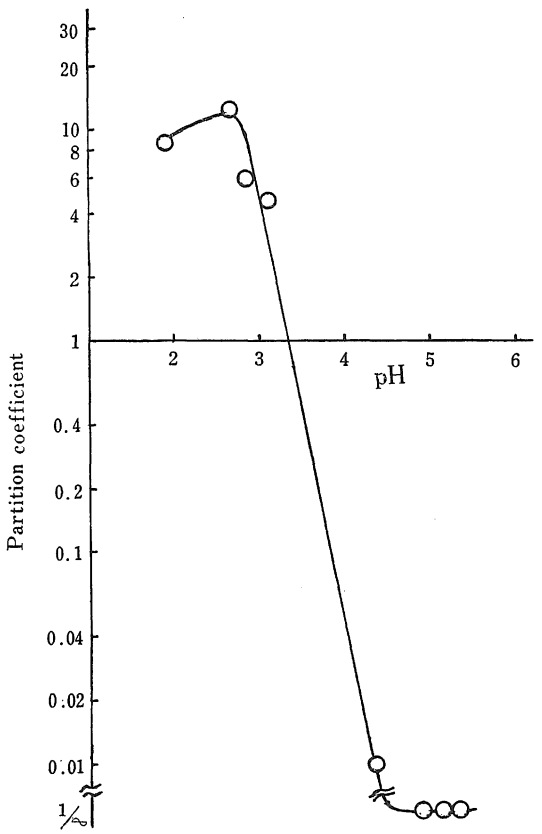

Fig. 1. Change of partition coefficient with changing pH

tained in BI−1 and BIII−1 have the same sedimentation constant, as seen in the Table. In contrast to these results, it was found that fractions separated by the precipitation method show definitely different solubility curves and different spectra, showing the existence of apparently different molecular species (6). From these facts, one possible way to account for the similar properties observed among the fractions is that the change of the partition coefficient

HMA monomer was isolated by Sephadex G−200 column and the chromatography was carried out using 1 g of this monomer. Although the same procedure as in the case of BPA was taken, a hard layer was formed at the top part of the column at early stage of the elution, which retarded the flow to a great extent. The hard layer may be caused from denaturation of HMA. From a much lower recovery of protein (~10%) in this case, most of the denatured protein may be considered to remain in the column. Obtained HMA fractions were combined into two fractions.

Fig. 3 shows solubility curves of BPA subfractions. Although it can be said that solubility curves most sharply reflect differences among the structures of plasma albumin subfractions, the curves of the BPA subfractions are very similar with each other and also to the curve of unfractionated BPA. Such is the case in HM A. Difference spectra between BI−1 and BⅢ−1 were observed by Cary Recording Spectrophotometer, in which study 0.3 % protein, 0.003 N NaCl solution was used ; however, it did not show any meaningful difference. Monomers con-

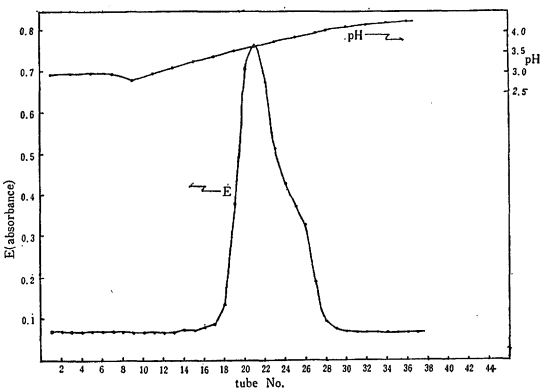

Fig. 2. pH gradient elution of BPA

* The monomer was checked with the centrifuge after dialysis and lyophilization.

Table

Fraction	pH range	yield mg	recovery %	dimer %	−SH content M/M	S
BI−1	3.40−3.60	21.0		28.7		4.3
BII−1	3.60−3.75	36.7	80.4	22.6		
BIII−1	3.75−4.00	22.7		16.5		4.3
deionized BPA Lot 9				17.5		
BPA Lot 9				9.0	0.70	
BI−2	3.50−3.65	237		20.6	0.16	
BII−2	3.65−3.75	169	72.0			
BIII−2	3.75−3.95	227		13.5	0.22	
BIV−2	3.95−4.10	87				

with changing pH might not be due to the normal N−F transformation. The pH region at which the partition coefficient changes with pH is approximately 0.5 unit lower than that of the usual N−F transition. The reason for this is not clear, but it might suggest that a somewhat differentt transition from the N−F transformation takes place as pH is changed.

The -SH content given in the Table are those measured by spectrophotometric method with p-chloromercuribenzoic acid(3) for monomers separated from BI−2 and BIII−2 by Sephadex column. These values are remerkably low compared with that of the starting material (Pentex Lot 9) which contains 9 % dimer.

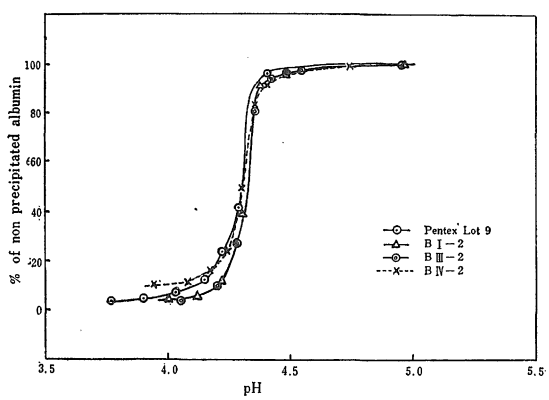

Fig. 3. Solubility behavior of BPA and its subfractions in 3 N KCL

During the chromatographic process, it may be considered that some change in molecular configuration takes place, besides dimerization. This change may be such that it buries -SH groups, but does not affect primary and/or secondary structures of the protein, since sedimentation constant and solubility behavior of the fractions are normal indicating undenatured properties of the protein. In other words, the carbitol-sucrose-water system might convert the tertiary structure of the proteins into a uniform configuration. This might be one of the reason why all the fractions have a similar character.

This work may be considered as the first application of pH gradient partition chromatography to a protein. In spite of the results reported here, the method seems still promising for separation of plasma albumin into subfractions, if an effort to find a good phase system is made, because of its good resolving power and reproducibility. From this point of view, the dextran-polyethylene glycol-water system

described by Albertsson(1) should be noted. In this system the partition coefficient of serum albumin changes with pH in the region of pH 3.0–5.0, which is exactly the same region as that of normal N-F transformation, although the change is less than that roported here.

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摘　　要

牛及び人血清アルブミンの小劃分を得るため，pH勾配分配クロマトグラフィーを行なった．分配相としては，ブチルアルコール，水，蔗糖を混合して得られる二相を使用し，支持剤としてセライトを使用した．

いわゆるN－F転移領域に相当するpH領域において，分配係数は大きな変化を示し，またその領域において，アルブミンが溶出した．得られた小劃分の性質を，溶解度曲線，超遠心法，示差スペクトル法，－SH含量等により比較したが，小劃分間に認めるべき差が見出せなかった．

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