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Effects of Long-Term Storage on Human Serum Albumin. II. Follow-up of Chromatographically and Ultracentrifugally Detectable Changes *

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This laboratory previously reported changes in normal human serum albumin stored under various conditions for 5 years. This report demonstrated that coordinated use of chromatography and ultracentrifugation offered a sensitive method for detecting protein alterations (1). In the present studies, which utilized the same method after another 5-year interval, the progress of these changes has been followed, and the relative stability of albumin stored under certain conditions has been determined.

Methods

The samples were vials of commercial normal human serum albumin, which was described previously (1). They had been prepared by low temperature ethanol fractionation of citrated whole plasma or ultraviolet irradiated dried plasma and had been heated at 60° C for 10 hours in the presence of stabilizers. Storage was at 5° or 32° C. The samples stored in the dry state had been sealed *in vacuo*.

Gradient elution chromatography of portions containing 500 mg of albumin was done at 6° C on 2.2- × 35-cm columns of the same diethylaminoethyl (DEAE) cellulose used in the earlier study (1). The gradient was from starting buffer (0.005 M H_3PO_4, 0.039 M Tris,[1] pH 8.6) to final buffer (0.5 M H_3PO_4, 0.5 M Tris, pH 4.1). The entire chromatographic procedure was that used previously (1), with the following exception. Whereas regeneration of the adsorbent had involved removing it from the column, washing with water, NaOH, water, warm ethanol, water, and starting buffer, followed by repacking, the present regeneration method consisted simply of flushing the column with approximately 400 ml of a solution that was 0.1 M in Na_3PO_4 and 0.2% (vol/vol) in Triton X-100 [2] (2) and subsequently flushing with at least 1,400 ml of starting buffer.

Appropriate chromatographic fractions were concentrated by negative pressure dialysis against 0.9% NaCl at 2° C, dialyzed against phosphate-buffered saline, pH 7.2, ionic strength 0.154, and centrifuged at 20° C in a Spinco model E ultracentrifuge at a protein concentration of 1.05%. They were then subjected to paper electrophoresis at room temperature in barbital buffer at pH 8.6. These procedures have been presented in detail previously (1); additional methods and any deviations from these procedures will be described below.

Elution patterns exhibited by the various samples have been classed into five general types, I through V (Figure 1), based on the typical chromatographic profiles shown in the earlier publication (1). Designations of plus or minus following the Roman numeral have been used to indicate the presence of more or less region 2 than that illustrated. In the present experiments, the small "foot" previously designated region 3 was usually not seen except in type I patterns; the small early peak seen in all patterns has been shown to be due to acetyltryptophan (1). It has been pointed out that changes in the chromatographic profile probably represent stages in a series of progressive alterations, rather than discrete steps (1). Furthermore, different pathways of alteration could lead to similar chromatographic patterns. Classification into types must therefore be regarded as a matter of convenience for grouping samples that have undergone a similar, but not necessarily identical, degree of change.

Results

Storage of lot 203, which was prepared from citrated whole plasma, at 5° C for as long as 10

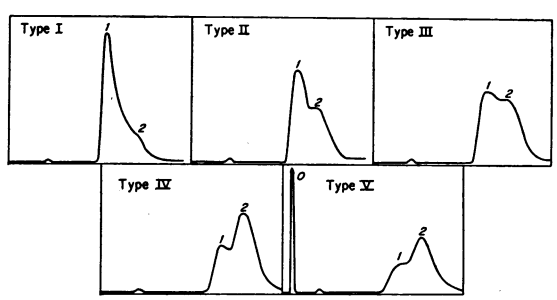

FIG. 1. CHROMATOGRAPHIC ELUTION PATTERN OF ALBUMIN SAMPLES. Abscissa is eluate volume; ordinate is absorbance at 280 mμ.

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[1] Tris = tris(hydroxymethyl)aminomethane.

[2] Triton X-100, Rohm & Haas Co., Philadelphia, Pa.

TABLE I

Summary of chromatographic patterns and ultracentrifugal analyses

Lot	Source*	Years stored	State†	Temperature	Chromatographic type	Sedimentation coefficients‡ Region 1	Sedimentation coefficients‡ Region 2
				° C			
				5	I	3.96	3.98, 5.71 (92%), (8%)
			5%	32	III	3.97	4.02, 5.68 (89%), (11%)
				5	I	3.96	4.03, 5.93 (75%), (25%)
203	CW	10	25%	32	IV−	3.99	4.05, 5.64 (80%), (20%)
				5	I+	3.96	4.04, 6.14 (80%), (20%)
			Dry	32	IV	4.07	4.52, 6.39, 7.62 (17%), (37%), (46%)
				5	II	4.03	4.03, 6.08 (70%), (30%)
			25%	32	IV	4.02	4.03, 6.18 (70%), (30%)
205	ID	10		5	II	3.94	4.06, 5.97 (55%), (45%)
			Dry	32	V	3.97	4.23, 5.96, 7.71 (25%), (33%), (45%)
				5	II	3.99	4.02, 5.84 (70%), (30%)
			25%	32§	IV−	3.97	4.05, 6.38 (77%), (23%)
201	CW	15		5	II		
			Dry	32§	V		

* CW = citrated whole plasma; ID = ultraviolet-irradiated, dried plasma.
† Stored as a 5 or 25% solution or as a freeze-dried preparation.
‡ Coefficients are s_{20} values reported in S units; figures in parentheses are relative proportions of the appropriate components.
§ Stored 5 years at 5° C, then 10 more years at 32° C.

years resulted in minimal change in the chromatographic pattern from that of fresh albumin, although the sample stored at this temperature in the dry state did yield a somewhat larger region 2. Albumin prepared from irradiated dried plasma and stored at 5° C for 10 years, or prepared from citrated whole plasma and stored at 5° C for 15 years, exhibited type II patterns (Table I).

The effect of the protein concentration on the chromatographic profile was pronounced in samples stored at 32° C. In the case of material from citrated whole plasma, storage as a 25% solution resulted in a less extreme type IV pattern than did storage in the dry state; storage as a 5% solution produced still less change, viz., a type III pattern (lot 203, Table I). To determine the effect of an intermediate temperature, a sample of lot 203 albumin that had been stored as a 25% solution at room temperature (approximately 25° C) was examined. The chromatographic pattern was of type III, demonstrating less severe deterioration than that of the comparable sample stored at 32° C.

In the other two lots, which exhibited type II patterns after storage at 5° C, storage at 32° C as

a 25% solution produced type IV patterns, whereas storage at this temperature in the dry state gave a pattern that has been designated type V (lots 205 and 201, Table I). The type V pattern was characterized by the presence of an extremely high, narrow "breakthrough" peak designated region 0 (Figure 1). Since this pattern appeared only in samples stored at 32° C in the dry state and had not been observed after 5 years of storage, it became desirable to examine lot 204, prepared from a mixture of citrated whole and dried, ultraviolet-irradiated plasma, the only lot from which a sample had been examined after 5 years of storage under these conditions (1). Accordingly, a sample of lot 204, which had been stored at 32° C in the dry state for 10 years, was chromatographed; it, too, revealed a type V pattern. Thus, of the samples from the four lots stored at 32° C in the dry state, only the one from lot 203 did not exhibit a type V pattern. Upon reconstitution of such samples from the four lots to a protein concentration of 10%, only the sample from lot 203 was not turbid upon visual inspection.

The fractions constituting region 0 were pooled, dialyzed against 0.9% NaCl, and measured volumetrically. Since these fractions were opalescent, protein concentration was determined by differential refractometry with the aid of a Brice-Phoenix instrument. Approximately 25% of the protein of samples showing type V patterns was found to be eluted in region 0.

The progression in the chromatographic patterns from type I to type IV involved not only the increase in region 2 at the expense of region 1, but also the successively later elution of region 1 itself. This trend, which was suggested by the data of the previous study, was confirmed in the present work. Although some of this effect could simply be due to the presence of less region 1 material, inasmuch as decreasing the size of a type I sample put on the column can result in later elution (3), two observations indicate that the differences are real. On a column of the size used in these experiments, decreasing the amount of the sample below 500 mg had little effect on the position at which region 1 was eluted (3); moreover, for a given lot and temperature, there was a tendency for region 1 of albumin stored as a solution to be eluted later than that of albumin stored in the dry state.

Upon ultracentrifugation, region 1 of all samples examined yielded a single peak with a sedimentation coefficient of approximately 4 S (Table I). On the other hand, region 2 from all samples, except those stored at 32° C in the dry state, gave two peaks during ultracentrifugation. The slower in each case had a sedimentation coefficient very near 4 S; the faster exhibited values varying from 5.6 to 6.4 S (Table I). A comparison of ultracentrifugal and chromatographic results shows that storage at 32° C brought about a considerable accumulation of the faster component. Storage of dry albumin at this temperature produced a still heavier component (7.6 to 7.7 S), which was the major ultracentrifugal peak in region 2 from these samples.

The effect of protein concentration on stability is apparent from the observations made on lot 203. Raising the concentration from 5% to 25% at 32° C resulted in the formation of a large amount of the 5.6 S component, and storage in the dry state, as reported previously (1), was even more deleterious (Table I). Even at 5° C, where much smaller changes in the chromatographic pattern occurred, stability was greatest at 5% concentration and least in the dry state. Similar differences were observed in albumins prepared from irradiated dried plasma, although these materials contained a greater proportion of the heavier components than did the corresponding samples from citrated whole plasma (lot 205, Table I).

Material stored as a 25% solution for 15 years showed relatively little change during the last 5 years (lot 201, Table I). Neither the chromatographic nor the ultracentrifugal results differed appreciably from those reported previously (1).

Region 0 from lot 205, which was prepared from irradiated dried plasma, was examined in the following manner. A sample of the fraction with the greatest absorbance at 280 mμ was dialyzed against phosphate-buffered saline, pH 7.2, ionic strength 0.154, and ultracentrifuged. The protein concentration determined by differential refractometry was 0.34%. Ultracentrifugation revealed a single peak skewed toward the bottom of the cell. The sedimentation coefficient calculated from the maximal ordinate of the schlieren pattern was 13 S. The remaining material from region 0 was then pooled. To avoid possible losses from adherence to the membrane during negative pressure dialysis, concentration was achieved by centrifuging for 10

hours at 35,000 rpm in the no. 40 rotor of a Spinco model L ultracentrifuge. The top 10 to 11 ml of each tube was drawn off with a syringe, and the remaining 1 to 2 ml was stirred, pooled, and dialyzed against the phosphate-buffered saline. Analytical ultracentrifugation of this preparation at a protein concentration of 1.05% gave results very similar to those obtained before concentration. Region 0 from lot 204, prepared from a mixture of citrated whole and irradiated dried plasma, was also concentrated by preparative ultracentrifugation. Upon analytical ultracentrifugation at a concentration of 1.05%, a single skewed peak which closely resembled that described above was observed. The sedimentation coefficient computed from the schlieren maximum was approximately 10 S.

Paper electrophoresis of region 0 showed a single broad band with mobility slightly less than that of albumin, but greater than that of α_1-globulin. This was true, regardless of whether or not the protein had been concentrated after chromatography. Because of a large amount of "tailing" (considerably greater than that usually seen for albumin) that could have obscured other components, region 0 was subjected to moving boundary electrophoresis at a protein concentration of 1.40%. This technique, carried out in an Aminco model B apparatus with barbital buffer, pH 8.6, ionic strength 0.1, at 0° C, revealed only a single, slightly asymmetrical peak, the mobility of which was indeed between those of albumin and α_1-globulin. On the other hand, paper electrophoresis of regions 1 and 2 yielded, in each case, a single band that migrated like the albumin in fresh plasma, an observation in agreement with that reported previously (1).

Discussion

The results presented above have demonstrated that, under proper conditions of storage, human albumin can be relatively stable for a period of 10 years. High temperature, even as high as room temperature, is to be avoided, as is storage in the dry state. Stability was somewhat greater at 5% protein concentration than at 25%, regardless of the temperature. The source from which the albumin is prepared is of considerable importance, as is evidenced by the fact that albumin prepared from irradiated dried plasma and stored at 5° C was chromatographically different from the comparable material prepared from citrated whole plasma. Moreover, there are differences in individual albumin preparations, even when the starting materials are similar. Lot 201, prepared from citrated whole plasma, exhibited a type II chromatographic pattern after 10 years of storage at 5° C (1), whereas lot 203, also prepared from citrated whole plasma, had a type I pattern after 10 years under the same conditions (Table I). Other such differences may be evident after shorter periods of storage.[3] Schmid, Polis, and Takahashi noted that certain albumin preparations displayed anomalous behavior at pH 4, and suggested that this might result from the removal of naturally occurring stabilizers during the isolation procedure (4). McMenamy has shown that the binding of indole analogues, such as tryptophan and the stabilizer acetyltryptophan, can vary with the treatment that the albumin has undergone, whether it be removal or replacement of bound fatty acids, or reworking to achieve further purification (5). Furthermore, Štokrová and Šponar have concluded that there is a statistical distribution of heat stability in populations of human albumin molecules (6, 7). Of two fractions these workers obtained by chromatography on DEAE cellulose, the one eluted earlier was the less heat stable (7). The degeneration of the chromatographic pattern from type I to type IV in the present work occurred at the expense of region 1.

Much of the change in albumin seen in our earlier and present work was of the nature of polymerization.[4] Similar changes have been observed by Pedersen, who subjected two samples of stored human albumin to gel filtration on Sephadex (8). These were not, however, the only changes occurring; changes in the elution position of region 1, which consisted entirely of monomer, have already been discussed. Moreover, in samples stored in the liquid state, the ma-

[3] A shorter-term study on other samples of human albumin prepared from fresh citrated whole plasma revealed that storage at 32° C for up to 35 months produced changes in the elution profile which, although detectable, were small, compared with those reported after 5 years at this temperature.

[4] "Polymerization" has been used to indicate the formation of dimers, oligomers, and higher aggregates, all of which have been designated "polymers."

jority of region 2 had a sedimentation coefficient of 4 S (Table I). Both of these findings indicate that, in addition to polymerization, considerable alteration of albumin monomer can occur during storage. This conclusion is in agreement with the observations of Pedersen and Andersson (8).

It is difficult to explain the properties of region 0. This material exhibited no binding to DEAE cellulose at pH 8.6, yet it migrated toward the anode during electrophoresis at this pH. Despite the fact that it was eluted in the "breakthrough" peak, it consisted entirely of high molecular weight components. Other polymers of albumin produced by ethanol denaturation (9), storage (reference 1 and Table I), or heating without stabilizers in the presence of citrate (3), have all been eluted later than region 1. The electrophoretic and ultracentrifugal properties of region 0 bore some resemblance to components formed by heating human albumin with inadequate stabilization or none at all (3, 10, 11). All samples in the present study, however, had been heated at 60° C for 10 hours before storage.

Summary

As a continuation of a previous report, chromatography on DEAE cellulose and subsequent ultracentrifugation were carried out on commercial preparations of normal human serum albumin stored under various conditions for at least 10 years. One lot of albumin prepared from citrated whole plasma and stored in solution at 5° C showed little difference in chromatographic behavior from fresh albumin. The storage of samples from this lot at 32° C produced large amounts of material that appeared later in the elution patterns and included components with sedimentation coefficients greater than 5.6 S. Regardless of temperature, albumin stored as a 5% protein solution underwent somewhat less change than that stored as a 25% solution and much less than that stored in the dry state. Samples prepared from ultraviolet-irradiated, dried plasma were less stable than those prepared from citrated whole plasma, irrespective of the storage conditions. Three out of four lots stored at 32° C in the dry state, the most deleterious storage condition found in this study, exhibited, in addition to the above changes, a component that did not bind to the DEAE cellulose and consisted of materials with sedimentation coefficients of 10 S or greater.

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