Amino Acid Substitution in Two Identical Inherited Human Serum Albumin Variants: Albumin Oliphant and Albumin Ann Arbor†

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ABSTRACT: Two inherited variants of human serum albumin, albumins Oliphant and Ann Arbor, have been examined in order to determine the amino acid substitutions. The albumins were purified by chromatography on DEAE-Sephadex from serum of affected members of the families. Tryptic digestions were carried out on the reduced and alkylated albumin and chymotryptic digests were prepared from heat denatured albumin Oliphant. Gel filtration, two-dimensional peptide mapping and ion-exchange chromatography were used to compare peptides from normal and variant albumin. The novel peptides thus detected were purified by further chromatography and their sequences determined. The region of interest in the variant was found to have the sequence Ala-Lys-Glu-Gly-Lys-Lys-Leu. The normal albumin had the corresponding sequence Ala-Glu-Glu-Gly-Lys-Lys-Leu. When the molecule was cleaved into two fragments by cyanogen bromide, the substitution was found in the C-terminal fragment. Albumin Ann Arbor was shown by peptide mapping to be the same variant. It is suggested that this albumin may be identical with several other variant albumins including albumin B which are generally of Scandanavian or north European origin.

Albumin Oliphant is an inherited, electrophoretic variant of human serum albumin which has been found in 22 members of a family of German descent, having the surname Oliphant (Adams, 1966; Weitkamp *et al.*, 1966). The transmission is autosomal and all affected individuals are heterozygotes. The serum of these heterozygotes yields two protein bands of equal intensity in the albumin region upon electrophoresis, one of which migrates cathodally to the normal component at alkaline pH. Family members with the variant seem to have no clinical problems related to the presence of the abnormal protein. Albumin Ann Arbor was similarly detected in an unrelated family of Danish descent (Weitkamp *et al.*, 1966).

Inherited electrophoretic variants of human serum albumin were first reported by Knedel (1957), Nennstiel and Becht (1957), and Earle *et al.* (1958, 1959). At least 15 different monomeric variants have now been distinguished by starch gel electrophoresis (Lie-Injo *et al.*, 1971) in addition to probably three different dimeric variants (Weitkamp *et al.*, 1971). However, little is known about the structural basis of this genetically determined variation. Previous efforts to identify an amino acid difference between normal albumin and a monomeric variant (Gitlin *et al.*, 1961) and normal albumin and a dimeric variant (Jamieson and Ganguly, 1969) by peptide mapping have not been completely successful. Gitlin and coworkers (Gitlin *et al.*, 1961) worked with a slowly migrating variant, albumin B, which is electrophoretically indistinguishable from albumin Oliphant and from 12 other variants found in presumably unrelated families (Weitkamp *et al.*, 1967), generally of northern European and Scandinavian ancestry (Weitkamp *et al.*, 1969). In albumin B two abnormal peptides were found after tryptic digestion and one after chymotryptic digestion. However, no normal peptides were shown to be missing. On this basis and considering the electrophoretic difference between the normal and variant albumin it was suggested that in albumin B a lysine residue had replaced a glutamic or asparatic acid.

In the present work albumin Oliphant has been purified and studied by conventional amino acid sequence technique in order to characterize its specific amino acid substitution. Since most of the amino acid sequence of human serum albumin is unknown, the strategy employed in this study was to carry out parallel experiments with normal and variant albumin. In this way, peptides from the abnormal albumin which had no counterpart in normal albumin were sought, as were peptides characteristic of the normal molecule but not found in the variant. Using tryptic, chymotryptic and cyanogen bromide digests of albumin Oliphant and albumin A, we were able to determine the sequence of a limited region of albumin and identify an amino acid substitution present in that sequence in the variant. Albumin Ann Arbor, previously supposed by us to be a distinct variant with similar electrophoretic properties, was shown to be identical with albumin Oliphant.

Methods and Materials

Blood samples were collected from affected family members by venipuncture without the use of anticoagulants. The blood was allowed to clot, the serum was decanted and the clot was then centrifuged to obtain additional serum. All serum samples were stored at −70° until used.

Chemicals. All chemicals were of reagent grade except as otherwise noted. Pyridine used in peptide chromatography was selected for low ninhydrin-reactive material and used without further purification. *N*-Ethylmorpholine was redistilled from ninhydrin (1 g/l.). Benzene used in the subtractive Edman degradations was redistilled. Ninhydrin, hydrindan-

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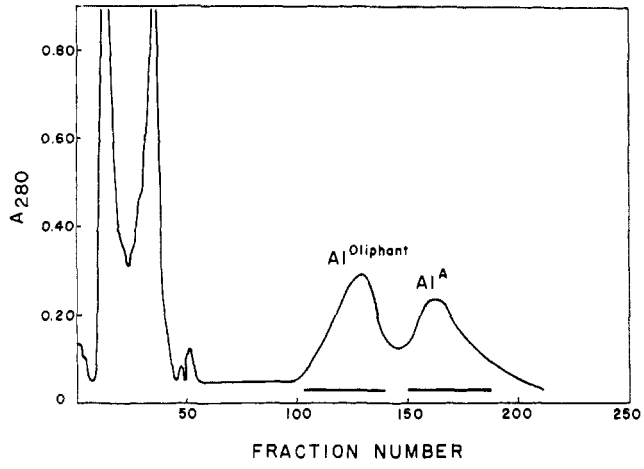

FIGURE 1: Chromatographic separation of Al^{Oli} and Al^{A} on DEAE-Sephadex at pH 5.75. Horizontal bars indicate regions that were pooled. For details of chromatography, see text.

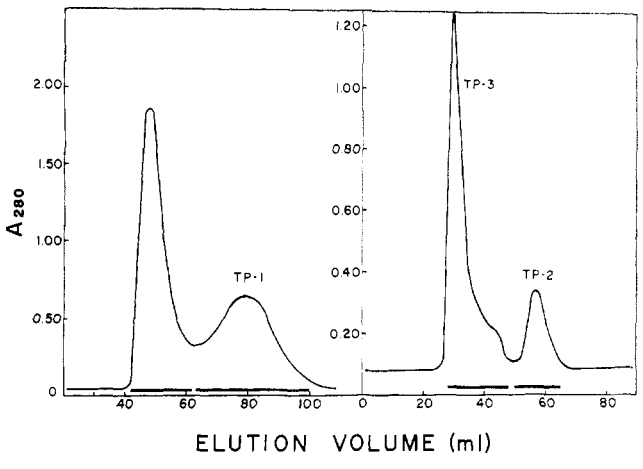

FIGURE 2: Subfractionation of tryptic digests of S-carboxymethylated albumin. (A) Chromatography on Sephadex G-10. Column dimensions, 1.5 × 80 cm. Solvent, 0.2 M NH_4HCO_3. Sample, 100 mg of albumin digest in 3 ml. Flow rate, 10 ml/hr. (B) Chromatography of the G-10 exclusion peak on Bio-Gel P-2. Column dimensions, 2.5 × 50 cm. Solvent, 0.2 M NH_4HCO_3. Sample volume, 2 ml. Flow rate, 20 ml/hr.

tin, and sequenator grade PITC[1] were purchased from Pierce Chemical Co. Trypsin, chymotrypsin and carboxypeptidases A and B were all products of Worthington Biochemical Corp. Crystallized HSA was obtained from Pentex Division of Miles Laboratories. Distilled water was deionized by passage through a mixed-bed ion-exchange column.

Amino Acid Analysis. Amino acid analyses were carried out by the procedure of Spackman *et al.* (1958) using a Beckman 120C automatic amino acid analyzer with a range expander to give a fivefold increase in sensitivity. Samples were hydrolyzed *in vacuo* in constant-boiling (105°) HCl sealed in Pyrex tubes. All buffers were purchased as concentrates from Himco, Inc., San Jose, Calif.

Peptide Mapping. Two-dimensional peptide maps were prepared as described by Bennett (1967). Samples were applied to Whatman No. 3MM filter paper sheets and chromatographed for 18 hr in 1-butanol–pyridine–acetic acid–water (90:60:18:72, v/v). Papers were air-dried overnight and subjected to high-voltage electrophoresis at pH 6.4 for 90 min at 2000 V in a Varsol-cooled Michl tank. Papers were developed by dipping in 0.1% ninhydrin in ethanol.

Sequence Determination. Subtractive Edman degradations were carried out by the method of Konigsberg and Hill (1962) using 60–120 nmoles of peptide. C-Terminal sequence determinations were carried out with carboxypeptidases A and B either separately or in mixtures (Ambler, 1967). Norleucine was used as an internal standard. The pH 2.2 sample buffer contained thienylalanine and aminoguanidinopropionic acid as internal volume standards. Samples of the reaction mixture were taken for amino acid analysis after 30-, 60-, 90-, and 180-min incubation at 37°.

Purification of Al^{Oli}. Serum samples were chromatographed on a 1.5 × 60 cm column of DEAE-Sephadex equilibrated with 0.2 M sodium phosphate buffer (pH 5.75). Samples of serum were prepared by diluting 4 ml of serum with 1 ml of phosphate buffer concentrate. The concentrate was prepared by dissolving sufficient NaH_2PO_4 to yield a 0.9 M solution and sufficient Na_2HPO_4 to yield 0.09 M solution. Upon fivefold dilution, the concentrations and pH of this buffer equalled that with which the column was equilibrated. The sample was applied to the top of the column by gravity; elution was carried out with the starting buffer throughout the run. Tubes were read at 280 nm in a Beckman DU spectrophotometer. The appropriate peaks were pooled, dialyzed against three changes of distilled water, and lyophilized.

Enzymatic Digestions. Albumin samples for tryptic digestion were reduced with a 20-fold molar excess (calculated on the basis of 17 disulfides/mole of albumin) of 2-mercaptoethanol in 8 M urea (pH 8.5). The reduced albumin was alkylated with a slight molar excess of iodoacetic acid in a Metrohm pH-Stat. Reagents were removed by passage through a Sephadex P-2 column in 0.2 M NH_4HCO_3. Tryptic digestions were carried out in 0.2 M NH_4HCO_3 (pH 8.5) at 37°. Trypsin was added at the start of the digestion, at 60 min and at 4 hr. After the last addition the incubation was continued for 16 more hr. The trypsin to protein ratio was 1:100 (w/w) at each addition. Protein concentration was 10–20 mg/ml.

Chymotryptic digestions were carried out by the method of Swaney and Klotz (1970). The albumin was heat denatured in dilute solution at low ionic strength and then sufficient solid NH_4HCO_3 was added to bring the concentration to 0.2 M. Chymotrypsin was added according to a schedule similar to that used for trypsin.

Cyanogen Bromide Treatment. Albumin Oliphant and albumin A were treated with a 30-fold molar excess of cyanogen bromide in 85% formic acid at room temperature for 30 hr (Gross and Witkop, 1962). The solution was concentrated *in vacuo* and applied to the top of a 3.5-m Sephadex G-75 column equilibrated with 8% formic acid. This column was constructed by completely filling three 1.5 × 90 cm columns and nearly filling a fourth column. These were then connected by narrow-bore tubing such that the bottom of the first column segment, the partially filled column, was connected to the bottom of the second segment. The top of the latter was connected to the top of the third segment and the bottom of the third segment to the bottom of the fourth. Effluent was collected from the top of the fourth segment; thus segments 1 and 3 flowed down ward while segments 2 and 4 flowed upward.

Automatic Peptide Chromatography. Peptide chromatog-

[1] Abbreviations used are: PITC, phenyl isothiocyanate; NEM, *N*-ethylmorpholine; HSA, human serum albumin; Al^{A}, normal albumin; Al^{Oli}, albumin Oliphant.

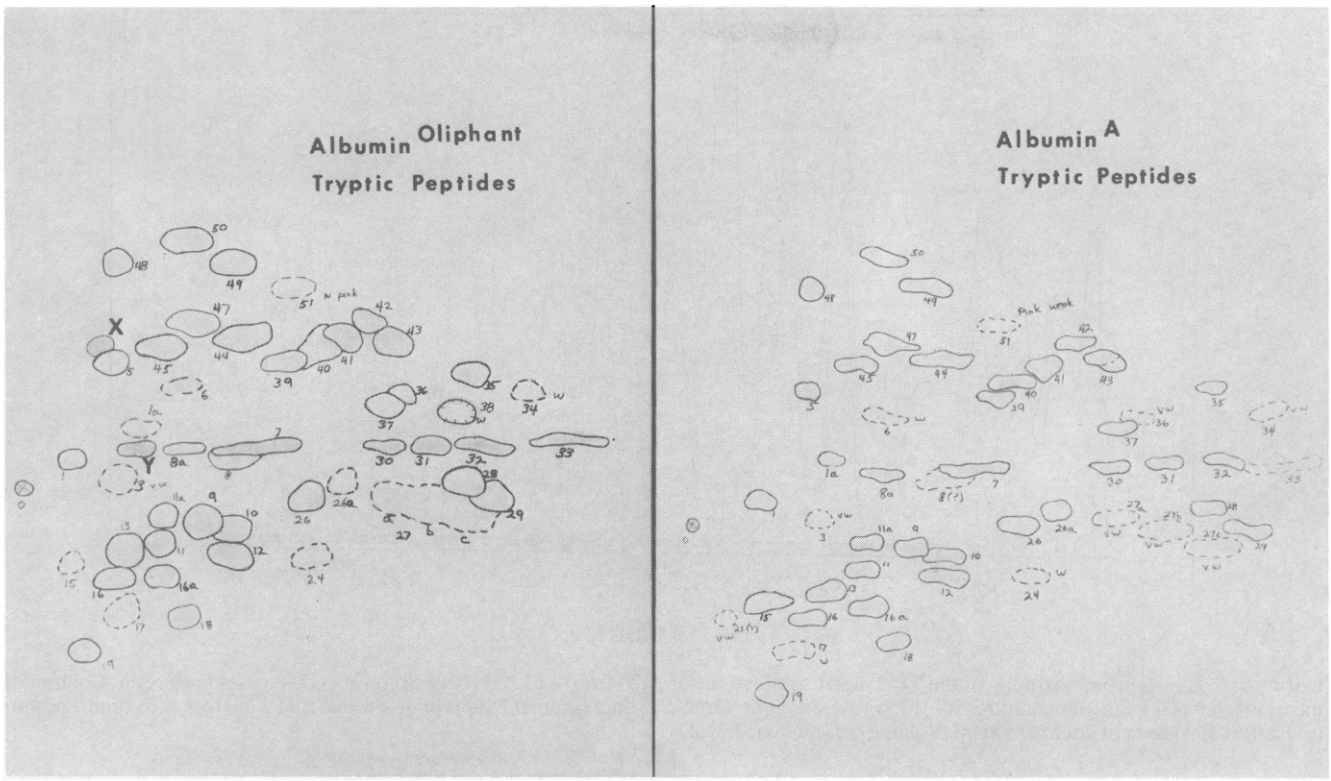

FIGURE 3: Two-dimensional paper peptide maps of the whole tryptic digest of S-carboxymethylated albumins Oliphant (A, left) and A (B right). The two new peptides in (A) are lightly shaded and designated X and Y. Descending chromatography from left to right, electrophoresis, cathode (top) and anode (bottom). For conditions, see text.

raphy was performed with automatic alkaline hydrolysis and ninhydrin development using an apparatus essentially identical with that described by Jones (1970). Ten percent of the column effluent was used for analysis and only the alkaline hydrolysis mode was employed. Pooled fractions were dryed *in vacuo* in round-bottom flasks in a rotary evaporator, dissolved in 2 ml of 0.1 M acetic acid, and stored in plastic vials. Purity of the peptide pools was assayed by high-voltage electrophoresis at pH 3.6.

Results

Purification of Al^{Oli}. The DEAE-Sephadex chromatography of Oliphant serum is shown in Figure 1. Pooled and lyophilized Al^{Oli} was assayed for purity by electrophoresis at pH 8.6 on cellulose acetate. In no preparation did contamination by Al^{A} exceed approximately 5% as judged visually from the Ponceau stained strips. Most preparations contained virutally no detectable Al^{A}.

Tryptic Digestions. Samples of Al^{A} and Al^{Oli} were reduced and alkylated as described above. Amino acid analysis was used to confirm the extent of the reaction. No Cys/2 was detected, even at sample loads ten times in excess of that required for normal analysis. The lyophilized *S*-carboxymethylalbumin was dissolved in 0.2 M ammonium bicarbonate and digested with trypsin at 37° for 20 hr. The long digestion period was used because the yield of C-terminal lysine and arginine as measured by carboxypeptidase B indicated that the digestion was more nearly complete than at 3–4 hr. The occurrence of chymotryptic peptides, a possibility in such a long digest period, was of no consequence in this work since normal and variant albumins were treated identically. The digest was concentrated *in vacuo* and applied to a Sephadex G-10 column (Figure 2A). The first fraction was similarly concentrated and chromatographed on Bio-Gel P-2 (Figure 2B). The fractions were designated TP-1, TP-2, and TP-3 as indicated in the figure. Under these conditions, any core that may have resulted remained soluble and would occur in TP-3.

Two-dimensional paper peptide maps of the main digest and each of the three digest subfractions were prepared by the methods cited above. Two new peptides were consistently found in the whole digest of Al^{Oli} (Figure 3). These were both detected in the TP-2 fraction. No normal spots appeared to be unequivocally absent in albumin Oliphant, although spot 15 was much diminished and was examined further as described below. A few spots, such as 33 and 38 (Figure 3), varied considerably from preparation to preparation but were found in both allotypes and were not considered further.

Al^{A} TP-2 and Al^{Oli} TP-2 were chromatographed on Beckman PA-35 ion-exchange resin using the automatic ninhydrin detection system described in the foregoing section. The separations were virtually identical except for the presence of a new peak in Al^{Oli} Tp-2 designated T2-16.9[2] (Figure 4). Each peptide pool indicated in Figure 4 by black bars was subjected to high-voltage electrophoresis at pH 3.6. By this technique, an additional abnormal peptide was detected in Al^{Oli} in peak T2-10. These pools were rechromatographed on Dowex 50-X2 as were the equivalent pools from Al^{A} TP-2. The new peptide

[2] Peptides are designated by a prefix letter T or C for tryptic or chymotryptic, respectively, a digit indicating the gel filtration peak and a second number, separated by a hyphen, which indicates the peak number in order of elution. If a peak has been rechromatographed, a lower case letter is used to designate the separated peptides. Novel peptides occurring in a chromatography are designated as a decimal fraction of the preceding normal peak. Roman numerals designate pool numbers in any given chromatography.

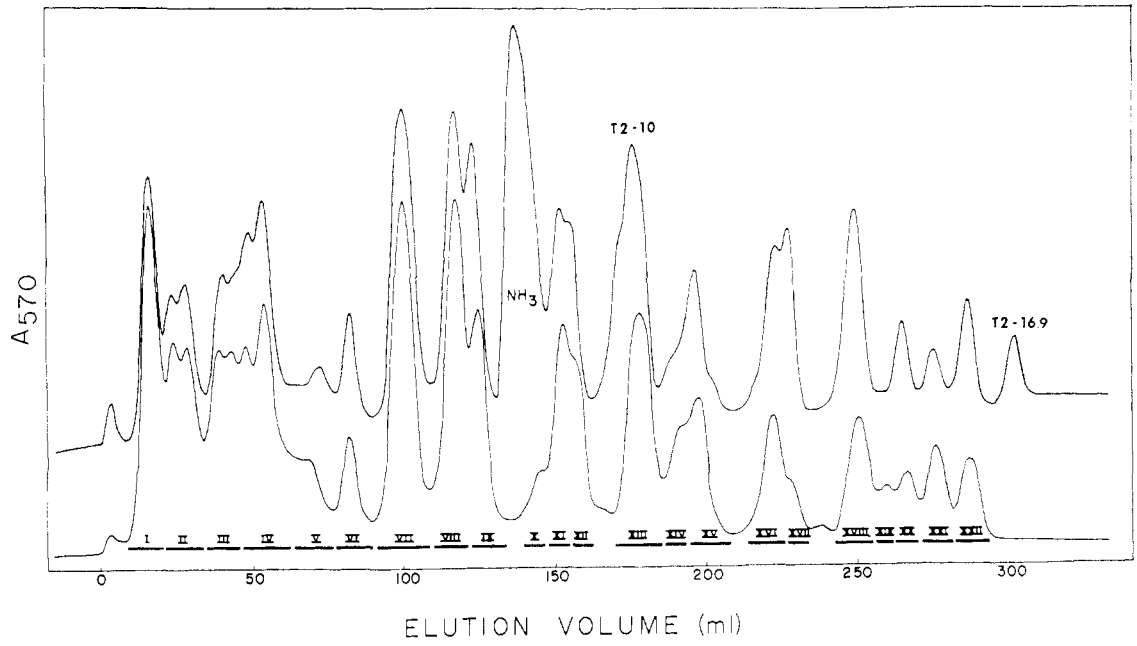

FIGURE 4: Column chromatography of the TP-2 digest subfractions of Al^{OIi} (top) and Al^{A} (bottom) on PA-35 ion-exchange resin. Column dimensions: 0.9 × 13 cm. Temperature, 50°. Flow rate, 30 ml/hr. Gradient, linear from 0.2 M pyridine–acetate (pH 3.04) to 2 M pyridine–acetate (pH 5.0). Total gradient volume, 500 ml. Volume per fraction, 2.2 ml.

TABLE I: Amino Acid Composition of Tryptic and Chymotryptic Peptides.[a]

	Al^{OIi}						Al^{A}							
	T2-9.9[b]		T2-16.9		C2-18.9		Cl-6-c		T3-18		Spot 15		T3-18a	
	A	R	A	R	A	R	A	R	A	R	A	R	A	R
Lys	9.4	1.0 (1)	8.3	1.9 (2)	14.1	2.8 (3)	81.8	2.2 (2)	50.6	2.6 (3)	11.8	2.0 (2)	NA	(1)
Asp									35.5	1.8 (2)	10.7	1.8 (2)		
Thr									15.2	0.8 (1)	5.7	0.9 (1)	21.6	0.9 (1)
Glu	9.0	1.0 (1)	4.7	1.1 (1)	5.4	1.1 (1)	75.8	2.0 (2)	56.5	2.9 (3)	18.5	3.2 (3)	68.4	2.8 (3)
Gly	9.0	1.0 (1)	4.6	1.0 (1)	5.2	1.0 (1)	31.2	0.8 (1)	18.3	0.9 (1)	7.1	1.2 (1)	27.5	1.1 (1)
Ala					5.5	1.1 (1)	38.4	1.0 (1)	39.0	2.0 (2)	11.2	1.9 (2)	26.8	1.1 (1)
Leu					5.3	1.1 (1)	46.0	1.2 (1)						
Phe									18.2	0.9 (1)	4.9	0.8 (1)	23.8	1.0 (1)
Total		3		4		7		7		13		12		8

[a] A = analytical results in nanomoles, R = residues per mole of peptide. Numbers in parentheses are assumed whole numbers. NA = not analyzed. [b] See footnote 2 for explanation of peptide numbering system.

was designated T2-9.9. The compositions of T2-16.9 and T2-9.9 are given in Table I.

Spot 15 (Figure 3) was found to occur in the TP-3 fraction in Al^{A}. Column chromatography of Al^{A} and Al^{OIi} TP-3 revealed the absence in the latter of a single peptide peak, T3-18. This peptide was purified by rechromatography on Dowex 50-X4. For comparison, the region containing spot 15 on the fingerprint was cut out and the peptide was eluted. The compositions of T3-18 and spot 15 were identical (Table I) except for a lower yield of lysine in spot 15. This is probably due to partial deamination of the ε-amino groups of the lysines by the ninhydrin reagent used to visualize the spots.

The sequence of the two tryptic peptides purified from albumin Oliphant and the one from albumin A are shown in Table II. The sequence of peptide T3-18 was determined by first conducting a carboxypeptidase A + B digestion to determine the C-terminal sequence. Since only one lysine was found in the C terminus, the peptide was digested with trypsin in NEM buffer (pH 7) to cleave it at the internal lysine. The reaction mixture was then applied as a line on prewashed Whatman No. 3MM filter paper, high-voltage electrophoresis was carried out at pH 3.6 and a guide strip cut from both sides was stained with ninhydrin. A new zone which was well separated from the other peptides was eluted with distilled water and analyzed. It was designated T3-18a and its composition is shown in Table I. Since the compositions of the fragments were nearly unique, the unfractionated digestion mixture was subjected to Edman degradation. After two turns further progress was blocked. However, it was evident even before that point was reached that T3-18 could not be related

TABLE II: Amino Acid Sequence of Tryptic and Chymotryptic Peptides from the Region of the Substitution.[a]

Peptide	Sequence
Al^{OII}	
T2-9.9[b]	Glu-Gly-Lys
T2-16.9	Glu-Gly-Lys-Lys
C2-18.9	Ala-Lys-Glu-Gly-Lys-Lys-Leu
Al^{A}	
C1-6-c	Ala-Glu-Glu-Gly-Lys-Lys-Leu
Al^{A}	
T3-18	Ala-Asx-Asx-Lys-Glu-Thr-(Glu_2,Gly,Ala)-Phe-Lys

[a] →, Edman degradation; ←, carboxypeptidase. [b] See footnote 2 for explanation of peptide numbering system.

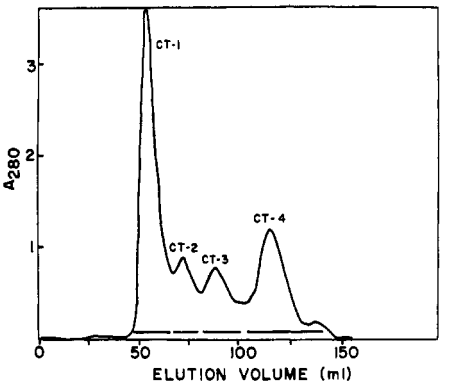

FIGURE 5: Subfractionation of chymotryptic digest of heat-denatured albumin by gel filtration on Bio-Gel P-2. For conditions see Figure 2.

to T2-16.9 and T2-9.9 by any common mutational process (Table II).

Chymotryptic Digestion. Al^{A} and Al^{OII} were heat denatured and digested with chymotrypsin as described under Methods. The digest, which was initially milky, cleared in about 5 hr and no insoluble material was detected. The digest was concentrated *in vacuo* and chromatographed by gel filtration on Bio-Gel P-2. Four subfractions were obtained, designated CT-1, CT-2, CT-3, and CT-4, in order of elution (Figure 5). Two-dimensional fingerprinting, done as before, showed a single abnormal peptide spot in albumin Oliphant CT-2 (Figure 6). Ion-exchange chromatography of the CT-2 subfractions (Figure 7) resulted in the detection of a basic peptide unique to albumin Oliphant, C2-18.9. No peptide was found to be absent from this subfraction, however. Peak C2-16 (Figure 7) which appeared to be missing in Al^{OII} CT-2 contained no demonstrable peptide material. Careful examination of the fingerprints of CT-1, -2, -3, and -4 revealed a single peptide spot occurring in Al^{A} CT-1 and weakly in CT-2 that did not appear to be represented in albumin Oliphant CT-1. The spot is seen as no. 4 in Figure 6. When the CT-1 subdigests were chromatographed, the absence of a peptide from Al^{OII} CT-1 was confirmed. The pooled fractions from albumin A CT-1 which contained this peptide were rechromatographed on Dowex 50-X2, and the purified peptide C1-6-c was analyzed (Table I) and subjected to sequence determination (Table II).

It can be seen that the abnormal chymotryptic peptide C2-18.9 is in excellent agreement with the tryptic peptides and permits the deduction of the sequence Ala-Lys-Glu-Gly-Lys-Lys-Leu from albumin Oliphant. Comparison to the normal sequence reveals a substitution to be Glu → Lys. That the residue is glutamic acid rather than glutamine may be inferred from the observation that the peptide is essentially isoelectric at pH 6.4.

Albumin Ann Arbor. While this work was in progress another abnormal albumin, albumin Ann Arbor, from a family which had been previously ascertained in this laboratory was examined (Weitkamp *et al.*, 1966). The albumin, which was electrophoretically indistinguishable from albumin Oliphant, was purified, reduced, alkylated and digested with trypsin. The digest was fingerprinted and the results are shown in Figure 8. It can be seen that the two abnormal peptides which are characteristic of albumin Oliphant are likewise present in albumin Ann Arbor, although spot 15 is not missing in Ann Arbor as it was in Oliphant. The presence of spot 15 probably is without significance since that peptide was shown to be unrelated to the substitution (*vide supra*).

Cyanogen Bromide Digestion. Al^{A} and Al^{OII} were treated with cyanogen bromide and concentrated *in vacuo* to 2-ml total volume for applications to the column. The absorbance of the fractions at 280 nm was determined and plotted as shown in Figure 9. Fragment F-I, F-II, F-III, and F-V were reduced and carboxymethylated as was done on the whole albumin. The modified fragments were digested with trypsin under the same conditions as were used when whole S-carboxymethylated albumin was digested and the digests fingerprinted as before. F-I from both Al^{A} and Al^{OII} gave a pattern which was nearly identical with *S*-carboxymethylalbumin of the corresponding allotype and probably represents uncleaved albumin. F-II is the large, C-terminal fragment of King and Spencer (1968) while F-III, F-IV, and F-V would correspond to their smaller N-terminal fragment. However, since the region is obviously heterogeneous, F-III and F-V were pooled and examined separately. F-IV, the overlap regions between F-III and F-V, was not treated further. F-III and F-V gave distinctive peptide maps but appeared to be extensively contaminated with each other. Both of the spots (X and Y) which characterized albumin Oliphant were found primarily in F-II.

Discussion

These results lead us to conclude that albumins Oliphant and Ann Arbor are the result of a point mutation leading to the substitution of a lysine for glutamic acid in the primary sequence. Since glutamic acid is coded by the codons GAA and GAG, the substitution must be G → A to yield a lysine codon AAA or AAG giving rise to the sequence Ala-*Lys*-Glu-Gly-Lys-Lys-Leu in place of the normal sequence Ala-*Glu*-Glu-Gly-Lys-Lys-Leu. This conclusion is based on the two abnormal tryptic peptides T2-9.9 and T2-16.9 and one abnormal chymotryptic peptide yielded by Al^{OII} C2-18.9 and on one normal chymotryptic peptide C1-6-C which was missing in Al^{OII}. In the variant there was an additional peptide missing (T3-18) which was apparently unrelated to the amino acid substitution. This may be a property of the albumin preparation itself or its degree of denaturation rather than the inherited sequence anomaly. Albumin Ann Arbor, which appears in all other ways to be the same substitution, is not deficient in this peptide.

Not all of the possible abnormal peptides were detected. Because of the Lys-Lys sequence, the region of interest in the

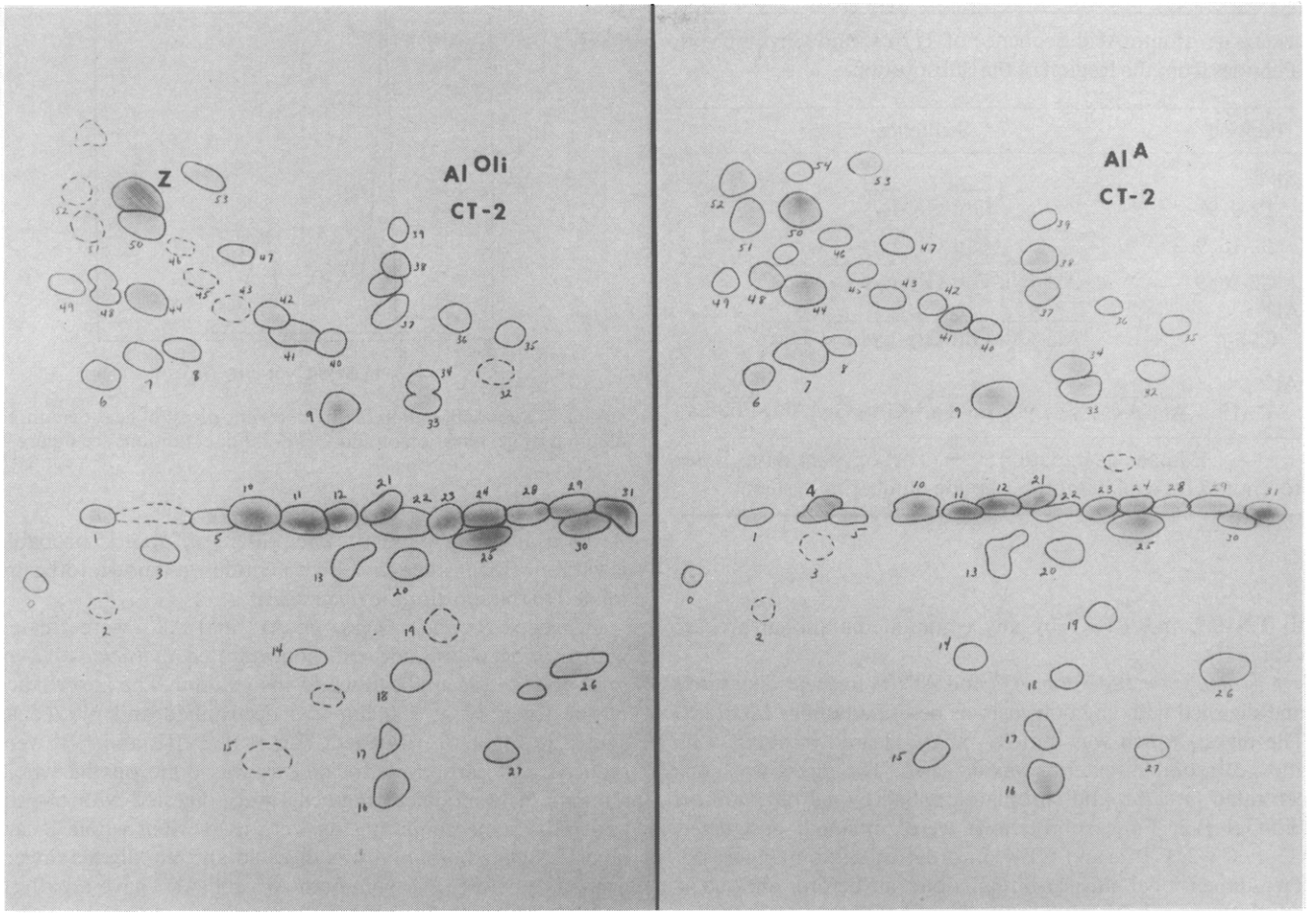

FIGURE 6: Two-dimensional paper fingerprints of subfraction CT-2 of Al^{Oli} (A) and Al^{A} (B). The abnormal and normal peptides in (A) and (B) are lightly shaded and labeled Z and 4, respectively. Descending chromatography from left to right, electrophoresis cathode (top), and anode (bottom). For conditions, see text.

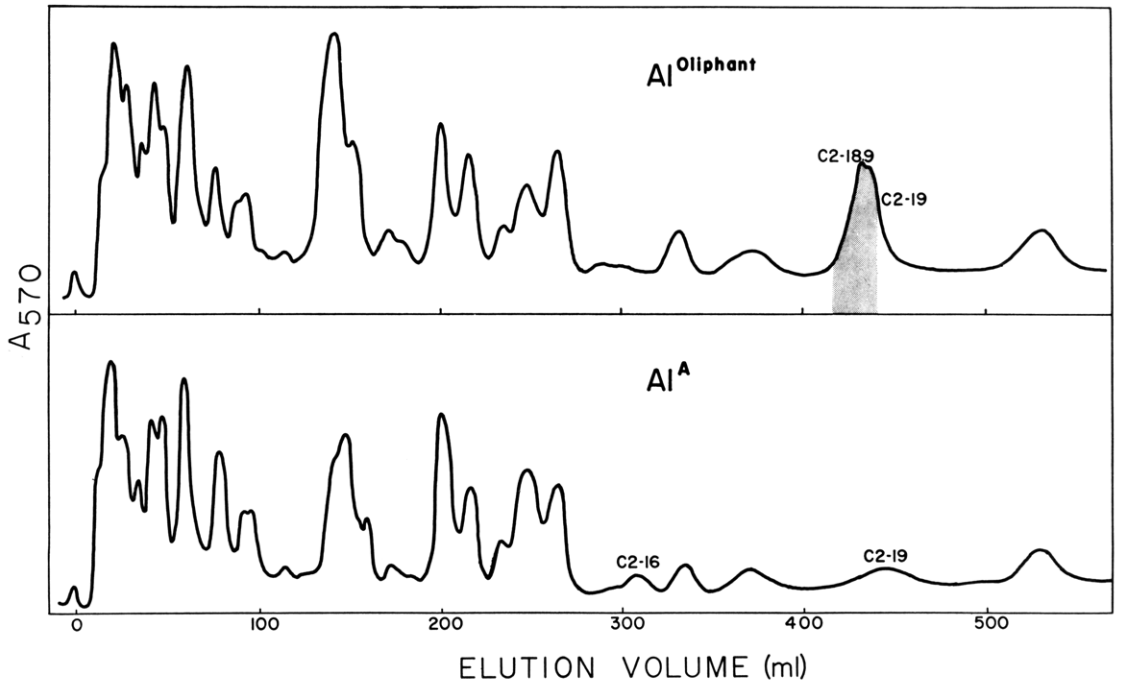

FIGURE 7: Column chromatography of the CT-2 digest subfractions on PA-35 ion-exchange resin. Conditions are the same as in Figure 4 except that at the conclusion of the gradient elution was continued with 2 M pyridine–acetate (pH 5.0) until an additional 75 ml had been pumped through the column.

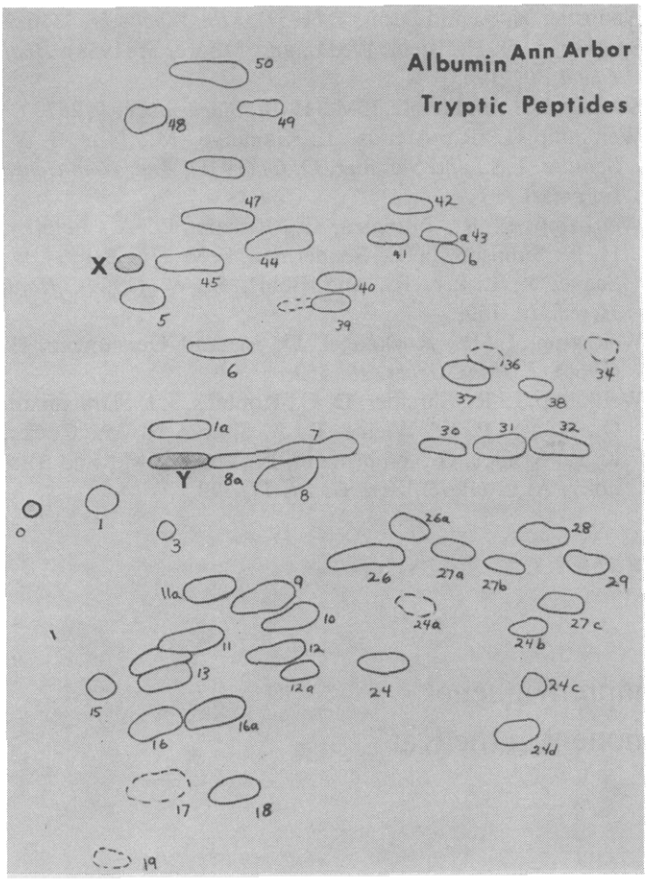

FIGURE 8: A two-dimensional fingerprint of the whole tryptic digest of S-carboxymethylated albumin Ann Arbor. The two new peptide spots are lightly shaded and designated X and Y. All conditions are the same as Figure 3.

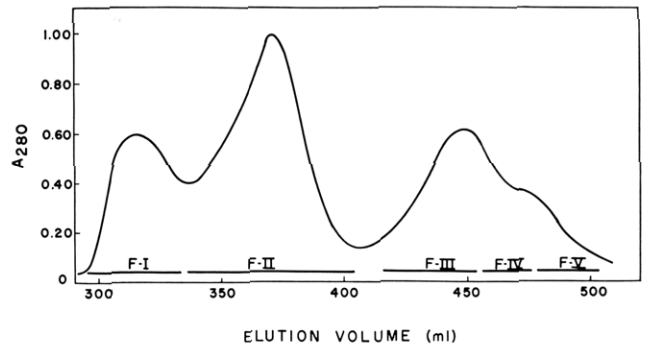

FIGURE 9: Gel filtration chromatography of the cyanogen bromide digest of native albumin on Sephadex G-75. Bars indicate regions under each peak that were pooled. For conditions, see text.

normal allotype should be represented in the tryptic digest by two peptides, one terminated by Lys, the other by Lys-Lys, and both of these should be missing in the variant. Similarly, the albumin Oliphant should contain, in addition to the two abnormal tryptic peptides observed, a third one terminated by the lysine which has been substituted for glutamic acid. None of these three peptides has been observed. Since all three contain a common sequence, namely that which is N terminal to the septapeptide described in this report, this absence may reflect some property of that peptide which makes it difficult to detect, such as low solubility under the conditions used in this study. In addition, if the peptide were very acidic it would not be chromatographically resolved on the Dowex 50-type resins. In regard to the possibility that the peptides in question are insoluble or nearly so, it was noted that ninhydrin-positive material was consistently observed on the origin of all fingerprints of whole digest or TP-3 peptides.

After determination of the nature of the amino acid substitution it became of interest to try to learn where in the molecule this substitution site was located. In order to do this, the albumins were treated with cyanogen bromide. Since King and Spencer (1968) have shown that cyanogen bromide cleaves native bovine serum albumin into two fragments, the variant was treated under these conditions in an attempt to determine which fragment contained the substitution. The finding of the abnormal peptide in the F-II fraction indicates that the site in question is in the C-terminal 75% of the molecule.

In the course of this work it was observed that the correlation between peptide size and behavior of the peptide on gel filtration of the digest was very poor. Moreover, since the digest of a protein this size should contain virtually a continuous distribution of peptide sizes, one should not expect gel filtration to yield a small number of discrete peaks. This leads us to suggest that at least some of these peptides associate with each other or with themselves and are chromatographed as aggregates on gel filtration. This is supported by the finding that nearly all of the acidic peptides in the digest chromatographed in the TP-3 fraction, irrespective of their size. The TP-3 fraction contained peptides which were excluded by both Sephadex G-10 and by Bio-Gel P-2 and should thus include only a few very large fragments. This behavior is consistent with the interpretation that the acidic peptides at least were strongly aggregated under the conditions used.

Albumins Oliphant and Ann Arbor have an electrophoretic mobility which is identical with albumin B and several other abnormal albumins which were independently ascertained from apparently unrelated families, all of Scandanavian or north European origin (Weitkamp *et al.*, 1967). In addition the migration of the two abnormal peptides in the peptide map of the tryptic digests of Oliphant and Ann Arbor is similar to the results obtained by Gitlin *et al.* (1961) in their study of albumin B. Unfortunately, differences in methodology make any detailed comparison of the peptide maps impossible. Nevertheless, these observations lead us to suggest that these two albumins are probably identical to albumin B and to at least some of the other north European "slow" albumin variants. Since albumins Oliphant and Ann Arbor are apparently identical, it seems unnecessary to maintain separate names for them. In view of the generally accepted practice of using geographical rather than personal names to designate protein variants, in the future we will use the name albumin Ann Arbor when referring to either of these two albumins.

If subsequent studies demonstrate conclusively the identity of albumin Ann Arbor and albumin B the latter name, by historical precedent, may be preferable.

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Isolation and Identification of 6-Methoxy-2-nonaprenylphenol as an Intermediate in the Biosynthesis of Ubiquinone-9 in the Rat*

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ABSTRACT: 6-Methoxy-2-nonaprenylphenol (6-MNPP) has been identified as an intermediate in the biosynthesis of ubiquinone-9 in rat liver. This metabolite has been purified from the neutral lipids of rat liver and analyzed by spectrometry. Its mass spectrum, ultraviolet absorption spectrum, and chromatographic properties correspond to those of an authentic synthetic specimen of 6-MNPP. It is enriched with radioactivity from benzoate-*U*-^{14}C, *p*-HBA-*G*-^{3}t, methionine-*methyl*-^{14}C, and mevalonate-*2*-^{14}C in liver slices engaged in the biosynthesis of ubiquinone-9 from these precursors. Synthetic 6-MNPP labeled in its methoxyl with tritium and administered intravenously was efficiently converted to hepatic ubiquinone-9 by intact rats.

In the rat, the aromatic ring of ubiquinone is derived from phenylalanine *via* tyrosine by a pathway that involves the transamination and dehydration of tyrosine to yield *p*-hydroxycinnamic acid, which then undergoes β-oxidation to form *p*-hydroxybenzoic acid[1] (Olson, 1966). In plants and microorganisms which synthesize the phenylamino acids, a branch point exists in the shikimate pathway which yields *p*-hydroxybenzoic acid (Rudney and Raman, 1966; Cox and Gibson, 1964; Whistance and Threlfall, 1967). *p*-HBA is thus a focal metabolite in ubiquinone biosynthesis in all organisms in which it has been tested. Argument now exists regarding the universality of the pathway from *p*-HBA to ubiquinone. In 1966, Friis *et al.* (1966) proposed a pathway for *Rhodospirillum rubrum* based upon the isolation of a number of prenylated phenols and quinones from lipid extracts of this organism although only two of the steps have been validated by biosynthetic studies for that organism (Raman *et al.*, 1969). Nonetheless, this scheme (Scheme I) (Nilsson *et al.*, 1968) has served as a useful working hypothesis for the investigation of the pathway from *p*-HBA to ubiquinone in a variety of organisms. Whistance and his collaborators (Whistance *et al.*, 1969–1971) have surveyed a large number of organisms for evidence of the presence of these postulated intermediates by chromatographic and radioisotopic techniques and have drawn tentative conclusions about the validity of the Folkers scheme for given organisms on the basis of the presence or absence of intermediates in the pathway. In some organisms capable of overall ubiquinone biosynthesis, most of the postulated intermediates could be detected although in others none was found. In *Pseudomonas ovalis*, they identified 2-polyprenyl-1,4-benzoquinone as an intermediate which appears "out of order" in the Folkers scheme. It is obvious that much further work must be done in order to exhaustively test this postulated sequence of reactions, including the ultimate identification of the enzymes involved.

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[1] Abbreviations used are: tlc, thin-layer chromatography; Q-9, ubiquinone-9; 5-DMQ-9, 5-demethoxyubiquinone-9; MVA, mevalonate; *p*-HBA, *p*-hydroxybenzoate; 2-NPP, 2-nonaprenylphenol; 6-MNPP, 6-methoxy-2-nonaprenylphenol; the *Chemical Abstracts* form of indicating isotopic labeling (^{14}C, *t*) is used.