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STRUCTURAL STUDIES OF TWO SERUM ALBUMINS
IN THE HOUSE MOUSE MUS MUSCULUS

BY

Ian A. MacLaren

A Thesis
Submitted to the Faculty of Graduate Studies through the
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ABSTRACT

Serum albumin (albumin A) from C3H mice and an electrophoretically slowly migrating variant (albumin C) from descendants of wild mice were extracted in ethanol from trichloroacetic acid precipitated serum and the monomer isolated by chromatography on Sephadex G-150. Preparations were judged free of non-albumin contamination by starch gel electrophoresis, analytical ultracentrifugation, exclusion chromatography and immuno-electrophoresis.

The normal and variant albumins exhibited identical extinction coefficients of 5.2 at 280 nm, sedimentation constants of 4.5 S, molecular weights as determined by SDS-polyacrylamide electrophoresis of 67,000 Daltons, similar amino acid compositions and complete serological identity. Microheterogeneity observed was due to albumin polymers as determined serologically, by their mobility in starch gels of increasing concentration, and the number of sulfhydryl groups (0.6) per molecule of albumin.

Peptide mapping of tryptic hydrolysates of the S-carboxymethyl albumin A and albumin C monomers revealed 3 variant peptides in the albumin C maps and one variant peptide in the albumin A maps. Elution of these peptides and amino acid analysis suggests that a glutamic acid residue in albumin A replacing a lysine residue in albumin C can adequately explain the mobility difference. The unexplained presence of a third variant peptide in the maps of albumin C may represent further variability in the variant molecule.

Also discussed was the feasibility of peptide mapping on samples from single animals.
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CHAPTER I

INTRODUCTION

Variation in human albumin was first reported by Scheurlen in 1955 (Peters, 1970) upon electrophoresis of serum from a diabetic patient. Since that time, at least fifteen electrophoretically distinct monomeric variants of human albumin have been detected in families or tribes from widely separated geographical areas (see Weitkamp et al., 1969; Lie-Injo et al., 1971). In addition, three different presumptive dimeric forms have been described (Weitkamp et al., 1972).

Studies on domestic mammals and birds have uncovered atypical forms of albumin in cattle, sheep, horses, pigs, chickens, turkeys, quail and doves (Peters, 1970). Pedigree analysis in humans and controlled breeding studies in animals have shown albumin variants to be generally under the control of codominant alleles at single autosomal loci. However Kristjansson (1966) has described a null allele in pigs which when homozygous, results in no detectable albumin being synthesized. Congenital absence of albumin or analbuminemia has also been found in man, but although these individuals are lacking a considerable fraction of their plasma protein, the condition is not associated with any clinical illness (Gitlin and Janeway, 1960).

In populations of wild mice albumin polymorphisms have been found to be widespread in both Peromyscus polionotus (Selander et al., 1971) and Peromyscus maniculatus (Brown and Welser, 1968).
However, in natural populations of *Mus musculus* only two electrophoretic variants, restricted in geographical distribution have been described. Albumin B was found as a double albumin in two animals from the northern region of the Jutland Peninsula, Denmark (Selander, 1970), and albumin C was found with normal albumin or albumin A in six mice of a sample of fifteen animals trapped near Windsor, Ontario. Breeding studies showed albumin A and albumin C to be controlled by two alleles at a single autosomal locus (Petras, 1972). No comparable studies with albumin B have yet appeared. Individuals of all inbred strains of *Mus* so far typed are homozygous for albumin A.

In spite of considerable variability in humans, domestic animals and rodents, only a few studies, aimed at determining the structural differences between the various albumin forms, have been reported.

Gitlin et al. (1961) sought support for a single amino acid replacement in their investigation of the cathodally migrating human albumin variant, albumin B. They concluded that a glutamic or aspartic acid residue in albumin A had been replaced by a lysine residue in the abnormal albumin B. Jamieson and Ganguly (1969) in their study of an inherited albumin dimer found no significant differences between the normal and abnormal albumins that would account for the variant's increased tendency to dimerize in vivo. In a more successful investigation of two slowly moving albumin variants of identical mobility, Winter et al. (1972), were able to conclusively demonstrate a substitution of a lysine for glutamic acid in the primary sequence of both variants. They also suggest that albumin B and the two variants, albumin Oliphant and Ann Arbor are identical.
Little appears in the literature on the purification and properties of mouse serum albumin. Popp et al. (1966) in their attempt to separate albumin from the so-called "albumin esterase" or esterase-1 reported amino acid analysis data on an albumin fraction eluted from DEAE-cellulose. Similarly, in their investigation of mouse alpha 1-antitrypsin, Myerowitz et al. (1972) reported two methods for the isolation of mouse serum albumin. Both of these studies were concerned with the purification and properties of molecules in the preparation of which mouse serum albumin was considered only a contaminant.

In the present study, normal mouse serum albumin and its electrophoretically slower variant, albumin C, were purified and characterized. In view of the central role of mice in numerous biological and biochemical investigations and the absence of any studies dealing specifically with mouse serum albumin, attention was paid not only to the differences between the normal and the variant forms but also to the behaviour and properties of mouse serum albumins as they compare with published data on the serum albumin of other species.
CHAPTER II

PURIFICATION OF ALBUMIN A AND ALBUMIN C

MONOMER AND POLYMER FRACTIONS

Albumin A and the variant albumin C were purified by trichloroacetic acid-ethanol extraction from serum. The monomers and polymers were isolated by chromatography on Sephadex G-150. Purity of the acid-ethanol and monomer preparations was determined by starch gel electrophoresis, sedimentation velocity analysis and immunoelectrophoresis.

Materials and Methods


    Serum for the preparation of albumin A was obtained from C3H mice (Alb-1\(^a\)/Alb-1\(^a\)). Homozygous (Alb-1\(^c\)/Alb-1\(^c\)) mice of stocks derived from the matings of wild (Alb-1\(^a\)/Alb-1\(^c\)) and C3H mice provided serum containing the variant or electrophoretically slow, albumin C (see Petras, 1972). In accumulating material for the isolation of the two types of albumin, blood from mice beyond 6 weeks of age were pooled.

    Capillary tubes were used to bleed the mice from the suborbital canthal sinus into 10 x 75 mm culture tubes which were refrigerated immediately after filling. The clot was allowed to retract for from 1 to 4 hours at 4 C then the blood was centrifuged at 1700 \(\times\) g for 15 min. The serum was removed and recentrifuged. Samples exhibiting
even mild hemolysis were discarded.

2. Trichloroacetic acid - ethanol extraction of albumin

The method of Iwata et al. (1968) with minor modifications was used for the initial extraction of albumin from serum. All steps were carried out at 4°C.

A sample of serum (4 ml) was diluted to 30 ml with distilled water then 10 ml of freshly prepared 20% trichloroacetic acid were added dropwise from a buret while the mixture was stirred constantly. The preparation was centrifuged at 25,000 x g for 10 minutes and the supernatant fluid was discarded. The sediment was resuspended in 3 to 5 ml of 5% trichloroacetic acid, brought to 40 ml with 5% trichloroacetic acid and centrifuged as before. The sediment was collected and resuspended in 3 to 5 ml of 80% ethanol, adjusted to 40 ml with 80% ethanol and set aside for 45 min. The ethanolic suspension was centrifuged at 25,000 x g for 30 minutes and the ethanol supernatant fraction, containing albumin was dialysed against several changes of distilled water and lyophilised. A second ethanol extraction of the trichloroacetic acid precipitate was omitted as preliminary work showed that this step contributed less than 1% to the total material recovered.

If quantities of serum other than 4 ml were extracted the ratio of final reagent volume to serum was always maintained at 10:1 (Iwata, et al., 1968).
3. Starch gel electrophoresis

Routine electrophoresis was carried out in 14% (w/v) starch gels (Connaught, Toronto), run at 4°C with a potential drop of 5 V cm⁻¹ across the gel. The gel and electrode buffers were 0.03 M and 0.3 M borate, pH 8.6, respectively (Petras, 1963). Best results were obtained when approximately 15 μl of 1.0% lyophilised protein dissolved in gel buffer was applied to each slot. Serum was usually diluted 1:3 with gel buffer. After electrophoresis gels were sliced horizontally and both halves stained with amido black.

4. Isolation of albumin A and albumin C monomers and polymers by

Sephadex G-150 chromatography

A 2.6 x 94 cm column (Pharmacia, Montreal) equipped with flow adapters was packed under gravity with Sephadex G-150 equilibrated with a 0.1 M Tris (tris-(hydroxymethyl)aminomethane)-HCl buffer, pH 8.1, containing 0.2 M NaCl (Pedersen, 1962). The continuity of the bed was checked by running Blue Dextran 2000, M.W. 2 x 10⁶ (Pharmacia) through the column. From 30 to 100 mg of the lyophilised ethanol-soluble material was dissolved in the Tris-HCl buffer and applied to the column in a 1-3% concentration. An upward flow was maintained by a peristaltic pump at 2.1 ml cm⁻² hr⁻¹ and 4 ml fractions were collected. Each fraction was read at 280 nm and the contents of each tube of the peak were pooled and concentrated by vacuum dialysis. These fractions were rechromatographed under the same conditions, concentrated then desalted on a 1.9 x 12 cm column of Biogel P-2 (Bio-Rad, Richmond) equilibrated with distilled water and lyophilised.
The purified monomer and polymers were then used without further treatment.

5. **Immunelectrophoresis**

Immunelectrophoresis was performed essentially according to Graber and Williams (1953) using albumin A and C monomer, ethanol soluble material and mouse serum. Rabbit antiserum to whole mouse serum (lot 5055) and mouse albumin (lot 5113) were obtained from Nutritional Biochemicals (Cleveland).

Electrophoresis was run in 1.5% (w/v) Difco Bacto Agar in 0.05 M barbital buffer, pH 8.6, for 3 hr at 4.8 mA per slide. The antigen wells contained 10 μl undiluted mouse serum and 10 μg of the monomer or ethanol-soluble material dissolved in 10 μl of 0.1 M Tris-HCl buffer, pH 8.1, with 0.2 M NaCl and 0.2 ml of antiserum were used undiluted. The precipitin lines were permitted to develop for 24 hr after which the slides were washed in saline for 48 hr, stained with 0.05% (w/v) amido black in 7% (v/v) acetic acid and destained in methanol:acetic acid:water (5:1:5).

6. **Ultracentrifugational analysis**

Ultracentrifugational analysis was performed in a Spinco model C analytical ultracentrifuge equipped with Schlieren optics with phase plate angle set at 60°. A 0.7% solution of the protein was run in 0.01 M Tris-HCl containing 0.32 M NaCl, pH 8.0 in an AN-H rotor at 56,000 r.p.m. at 20 C.
Results and Discussion

1. Trichloroacetic acid-ethanol extraction of albumin and Sephadex G-150 chromatography

Numerous procedures have been used for the purification of serum albumin including the low temperature alcohol fraction techniques of Cohn et al. (1947, 1950), starch block electrophoresis (Allerton et al. 1962), DEAE-cellulose chromatography (Popp, 1966) and molecular exclusion chromatography (Flodin and Killander, 1962).

In the present study for the isolation of the normal (A) and variant (C) albumins, advantage was taken of the property of albumin to redissolve in ethanol after its precipitation from serum (together with the other proteins) by 5% trichloroacetic acid (Koronera and Debro, 1956). The method of Schwert (1957) as modified by Iwata et al. (1968) resulted in a clean "one step" purification of mouse serum albumin although formation of albumin polymers did occur. After dialysis and lyophilisation of the ethanol supernatants from trichloroacetic acid precipitated C3H serum and serum of homozygous albumin C mice, 30 to 35 mg of material was recovered per ml of serum processed.

Figure 1 shows the results of electrophoresis of these two fractions at pH 8.6. Each sample showed a prominent anodally migrating band which corresponded in mobility to the albumin band of the serum from which it was isolated, and a series of from 2 to 4 trailing bands with mobilities showing no correspondence with any components of whole serum, yet consistently reflecting the mobility difference
Starch gel electrophoresis at pH 8.6 of albumin A and albumin C trichloroacetic acid-ethanol extract and Sephadox G-150 fractions I and II.

Albumin A (C3H) serum (a), albumin C serum (b), trichloroacetic acid-ethanol extract of albumin A (C3H) serum (c), trichloroacetic acid extract of albumin C serum (d), G-150 fraction II of albumin A (e), G-150 fraction II of albumin C (f), G-150 fraction I of albumin A (g). Lyophilised protein, stored for several months at -4°C was redissolved at 1.0% in gel buffer (0.03 M borate, pH 8.6), and 15 μl applied to each slot. Serum was diluted 1:3 with gel buffer.
exhibited by the albumin A and C monomers. Since it was not clear as to whether these bands represented aggregates of mouse serum albumin monomer or complexes of albumin with other serum proteins or peptides (Ballieu and Imhof, 1962) the monomer and trailing material were separated for further independent characterization (CHAPTER III). The need for this step was reinforced by the fact that a protein of high purity is required for structural studies (Needleman, 1970) and the abnormal albumin C was originally described in serum as a monomeric variant (Petras, 1972).

Molecular exclusion chromatography on Sephadex G-150 succeeded in isolating the monomer from the heavier material which appeared in earlier fractions and constituted approximately 10% of the total protein applied. Figure 2 shows a typical chromatogram of the ethanol-soluble albumin A material as eluted from G-150 in the Tris buffer. Chromatograms of albumin C were identical in all respects. Starch gel electrophoresis showed the albumin monomer to be present in fraction II whereas fraction I was enriched with material corresponding to the trailing bands. The elution pattern presented in Figure 2 is similar to that obtained by Pedersen (1962) in his study of bovine albumin aggregates on G-150 where the peak preceding the monomer could be shown by ultracentrifugation to be the albumin dimer and earlier peaks, higher polymers of albumin.

Since cross-contamination between fractions I and II was apparent on electrophoresis they were again chromatographed individually on G-150 as before. Fraction II eluted as a single symmetrical peak while fraction I was resolved into two adjacent and overlapping peaks,
Fractionation of trichloroacetic acid-ethanol albumin A extract on Sephadex G-150 column.

Forty mg of 1.0% albumin A trichloroacetic acid-ethanol extract dissolved in 0.1 M Tris-HCl buffer, pH 8.6 containing 0.2 M NaCl were applied to a 2.6 x 94 cm column of Sephadex G-150. An upward flow of the same buffer was maintained with a peristaltic pump. Fractions of about 4 ml were collected and read at 280 nm. Fractions were pooled as indicated and concentrated by vacuum dialysis, desalted and lyophilized. Chromatograms of albumin C material were identical.
the first exhibiting a maximum at 280 nm in tube 7 (Ve 185 ml) and
the second in tube 11 (Ve 205 ml). Electrophoresis of the two
fractions after rechromatography showed only barely detectable cross
contamination, however upon lyophylisation and storage of the monomer
for several months at -4 C the additional bands reappeared. Similarly,
upon storage of the material of fraction I, a faint band corresponding
in mobility to the monomer also reappeared. As with preparations of
bovine and human albumin purified by ethanol fractionation (Cohn et
al., 1947), the behaviour of these components during low temperature
storage is consistent with the view that the heavier material repre-
sents albumin aggregates (Saifer et al., 1961; Janatova et al., 1968).

2. Criteria of purity

Before a structural analysis of any protein is initiated, a
preparation of high purity must be available. Experiments performed
with contaminated material will invariably lead to erroneous con-
cclusions (see discussions by Blackburn, 1970 and Holde, 1970). A
protein homogeneous by one criterion may reveal complex heterogeneity
when examined by other techniques exploiting different characteris-
tics of the molecule. There are few proteins that better illustrate
the nature of this problem than serum albumin.

Two general categories of heterogeneity can be distinguished
using preparations of serum albumin. The term macroheterogeneity refers
to contamination by proteins other than albumin and the term microhetero-
genity involves molecular subspecies of albumin as distinguished by
electrophoresis (Saifer et al., 1961, Janatova et al., 1968) ion-
exchange chromatography (Shrivastava et al., 1972), isoelectrofocusing (Spencer and King, 1971), and salt precipitation (Wong and Foster, 1969; Sogami et al., 1969). This latter form of heterogeneity appears to arise from post-translational modifications leading to the binding of fatty acids, formation of polymers and mixed disulfides involving the single free SH group common to all albumins and to intramolecular disulfide interchanges. There is no evidence that albumin molecules of different primary structure exist in a single individual with the exception of allelic variants.

Since the main concern in this study is the variability in the primary structure, a sufficient criterion of purity is the absence, in monomeric preparations, of contamination of non-albumin origin.

Apart from the trailing bands in both the A and C ethanol-soluble and monomer preparations, no evidence for contamination by other serum proteins could be found. As pointed out previously the third trailing band of the alcohol-soluble material closely corresponded in mobility with a component of whole serum upon electrophoresis (Figure 1). This band which stained very lightly had a mobility which appeared very similar to that of transferrin and raised the possibility of contamination by this serum protein. However, electrophoresis of the albumin A alcohol-soluble material from CBA mice whose serum contains a faster migrating transferrin component (Shreffler, 1960) than found in serum of C3H or mice with albumin C, showed no increased mobility of the third band and thus ruled out homology with transferrin.

Figure 3 represents the sedimentation velocity pattern of the
Figure 3

Sedimentation velocity pattern of albumin A and albumin C fraction II (monomer).

Sedimentation velocity analysis was carried out on 0.7% albumin A and albumin C fraction II dissolved in 0.01 M Tris-HCl, pH 8.0 containing 0.32 M NaCl. The photograph was taken 128 min after reaching maximum speed of 56,000 rpm.
direction of sedimentation
albumins A (upper) and C monomers (lower) 120 min after reaching speed in the ultracentrifuge. An asymmetrical leading edge of the peak was observed indicating contamination by heavier material, perhaps the molecules responsible for the slower bands on electrophoresis.

Immunelectrophoresis of alcohol-soluble A and C preparations (Figure 4) when developed with anti-whole mouse serum antiserum showed the material to be devoid of any obvious non-albumin contamination. Immunelectrophoresis of the A and C monomer preparations against the same antiserum revealed an identical pattern suggesting that with respect to non-albumin contamination, no further purification was obtained by chromatography on G-150. The components above the major albumin precipitin arcs in Figure 4 were judged to be of albumin origin as they also appeared when anti-mouse albumin antiserum was used in place of whole serum antiserum.

The presence of the heavier material in ethanol-soluble albumin extracts and the reappearance of these forms on electrophoresis after lyophilisation of the albumin A and albumin C monomer fractions purified by G-150 rechromatography provides evidence that neither of these preparations are homogeneous. However in adopting as a criterion of purity the absence of any non-albumin contamination, the probability that foreign proteins present in the preparations would escape detection is greatly reduced and the suitability of the material for further structural work can be readily determined. On the basis of charge, molecular size and especially immunochemical reactivity no evidence of non-albumin contamination could be demonstrated in either
Figure 4

Immunelectrophoresis of albumins A and C trichloroacetic acid-ethanol extracts and G-150 fractions I against rabbit antisera to whole mouse serum and mouse albumin.

The samples are albumin A trichloroacetic acid-ethanol extract and C3H serum (a), albumin C trichloroacetic acid-ethanol extract and albumin C serum (b), albumin A fraction II (monomer) and C3H serum (c), albumin fraction II (monomer) and albumin C serum (d). Antigens in (a) through (d) precipitated with whole mouse serum antisera. Albumin A (upper) and C (lower) fraction II (monomer) precipitated with mouse serum albumin antisera (e). Lyophilised protein (0.1%) dissolved in 0.1 M Tris-HCl buffer, pH 8.6 containing 0.2 M NaCl was present in each antigen well and antisera was used undiluted. Slides were developed for 24 hr, washed and stained with amido black.
the alcohol-soluble or monomer preparations of albumin A or albumin C. The fact that the trailing bands did not correspond to any components of whole serum (as judged by starch gel or immunoelectrophoresis in the absence to molecular sieving), yet reflected the mobility difference of their respective (A or C) monomers and could be precipitated by specific antisera directed against mouse serum albumin suggest that these forms represent microheterogeneity within the albumin preparations and therefore are albumin in origin. This latter conclusion is further substantiated in the next chapter.
CHAPTER III

CHARACTERIZATION OF ALBUMIN A AND ALBUMIN C

MONOMER AND POLYMER FRACTIONS

In order to assess the similarity between mouse serum albumin and albumins of other mammalian species and to detect any differences between albumin A and the variant albumin C, several physicochemical properties were investigated. The extinction coefficient, sulfhydryl content, sedimentation constant, molecular weight, amino acid composition and serological identity of the albumin A and albumin C monomers were determined. The nature of the trailing bands and material of higher molecular weight isolated on G-150 were investigated in starch gels of increasing concentrations and by double immunodiffusion against mouse serum albumin antiserum.

Materials and Methods

1. Determination of the extinction coefficient and measurement of protein concentration

The extinction coefficient (\( \text{OD}_{1cm} \), the optical density of a 1% solution of protein where the light path equals 1 cm) was determined on weighed, salt-free lyophilized samples of albumin A and albumin C monomer dissolved in distilled water using a Coleman Hitachi (Perkin Elmar) model IIII spectrophotometer at 280 nm. Optical densities were corrected for moisture content by reweighing weighed samples of.
protein dried at 105 C overnight. Weighings were performed on an
electrobalance (Cann model G-2, Vantron, Paramount).

All determinations of albumin concentration for mouse albumin
were made either by the weighing of lyophylised material or by an
\[ \frac{1}{\text{cm}} \]

\[ \text{cm} \] of 5.2 at 280 nm.

2. Immunodiffusion

Immunodiffusion (Quinlan, 1968) was performed in order to
determine the degree of immunological identity between albumin A and
albumin C monomer and between components from the heavier G-150
fraction I material. Slides were prepared with 1.5% Difco Bacto Agar
dissolved in 0.15 M NaCl. Twenty \( \mu l \) of 0.1% antigen and 20 \( \mu l \) of
undiluted antiserum were used in all plates. Preparation of the pro-
tein solutions, conditions for diffusion and staining of the slides
were the same as those described earlier for the immunoelctrophoresis
(CHAPTER I).

3. Sulphydryl determinations

Free thiol groups of the albumin A and C monomers were determined
by the method of Janatova et al. (1968) using 5-5'-dithiobis
(2-nitrobenzoic) acid (DTNB) (Aldrich).

A DTNB stock solution (0.01 M) was made up in 0.050 M phosphate
buffer, pH 8.0. A reagent blank was prepared by mixing 0.20 ml of the
stock DTNB solution with 0.05 ml of 0.025 M ethylenediaminetetraacetic
acid (disodium EDTA dissolved in 0.0074 M phosphate buffer, pH 8.1)
and 0.75 ml of 0.0074 M phosphate buffer, pH 8.1, in a 1.0-ml cuvette.
The reaction mixture was identical except that 0.4% solutions of albumin A and albumin C were made by dissolving a carefully weighed sample of the lyophylised monomer in 0.0074 M phosphate buffer, pH 8.1. Forty-five minutes after mixing, the absorbance of the reaction mixture was analyzed at 412 nm against the reagent blank in the spectrophotometer. An albumin blank was prepared by mixing 0.75 ml of the albumin solution with 0.2 ml of 0.050 M phosphate buffer, pH 8.0 and 0.050 ml of 0.025 M EDTA. The absorbance of this solution (at 412 nm) was subtracted from the absorbance of the reaction mixture. A molar extinction coefficient (E_{412} nm) of 13,600 M^{-1} cm^{-1} was found for the thionitrobenzoate anion upon standardization with cysteine (free base, Mann Research Laboratories) assuming a single mole of SH groups per mole cysteine.

4. **Determination of sedimentation constant S_{20,w}**

The sedimentation coefficient of the albumin A and albumin C monomer was determined by sedimentation velocity analysis in the Spinco Model C ultracentrifuge under conditions described in **CHAPTER I**. Photographs were taken at 16 min intervals and S_{obe} was corrected for the density of solvent when calculating S_{20,w}.

5. **Molecular weight determination by SDS-polyacrylamide electrophoresis**

Molecular weights of the albumin A and albumin C monomers were determined using the method of Weber and Osborn (1968).
All samples were dissolved in pH 7.0, 0.01 M sodium phosphate buffer, containing 0.1% sodium dodecyl sulphate (Sigma) and 0.1% 2-mercaptoethanol (Sigma) 24 hr before determination. The gels were run for 4 hours at 8 mA per tube. After electrophoresis gel length and the distance of migration of the bromophenol blue were measured and the gels stained in Coomassie brilliant blue, destained and the migration distance of the stained protein bands were remeasured. Ribonuclease A, M.W. 13,700; chymotrypsinogen A, M.W. 25,000; ovalbumin, M.W. 45,000 and bovine serum albumin, M.W. 68,000 were run as standards.

6. Electrophoretic analysis of the trailing bands

Electrophoresis of the G-150 fraction I components showed them to correspond to the trailing bands in the ethanol-soluble preparations (Figure 1). In order to determine if these bands represent albumin aggregates, their behaviour in the presence of 2-mercaptoethanol and the dependence of their electrophoretic mobility on increasing starch concentration was investigated.

Samples of the ethanol-soluble material and G-150 fraction I were run in starch gels containing 0.015 M 2-mercaptoethanol. About 18 hr before electrophoresis the albumin samples were dissolved in gel buffer containing the same concentration of 2-mercaptoethanol.

The mobilities of the albumin A and albumin C monomer and trailing components relative to bromophenol blue were measured in 12, 14, 16 and 18% (w/v) gels. Conditions for sample preparation and electrophoresis were identical to those for routine starch gel
7. Preparation of reduced and carboxymethylated albumin

The alcohol-soluble and purified monomer preparations of albumins A and C were reduced and alkylated by the method of Crestfield et al. (1962). However, reduction and alkylation were carried out for 24 hr and 40 min, respectively.

From 25 to 50 mg of protein were placed in an airtight 12-ml screw-cap vial maintained under a nitrogen barrier and to this were added 3.61 g of deionized, crystalline urea (urea was further purified by dissolving in deionized water and stirring overnight with AG-50X8 mixed bed ion-exchange resin (BioRad) and then recrystallizing from water at 60°C), 0.30 ml of EDTA solution (50 mg of disodium EDTA per ml), 3.0 ml of Tris buffer, pH 8.6, (5.23 g of Tris and 9 ml of 1.0 N HCl diluted to 30 ml with water), and finally 0.10 ml of 2-mercaptoethanol. The solution was made up to a 7.5 ml mark with water, and a solution of 8 M urea and 0.2% EDTA was used to fill the vial completely. After 24 hr at room temperature, the contents of the vial were transferred to a 25-ml beaker under a nitrogen barrier. A freshly prepared solution of 0.268 g of iodoacetic acid in 1.0 ml of 1.0 N NaOH was added to the reaction mixture. The reaction was stirred and allowed to proceed for 40 min in the dark then 1.0 ml of 2-mercaptoethanol was added to titrate the excess iodoacetic acid. The mixture was then exhaustively dialysed in the cold and lyophilised.

The extent of alkylation was determined by amino acid analysis.
8. Amino acid analysis

Lyophilised, S-carboxymethylated samples of albumin A and albumin C monomer and trichloroacetic acid-ethanol material were weighed out and transferred to 10 ml ampules. HCl(6N) containing 0.2 μmoles norleucine per ml was added to each ampule to a final concentration of 1 mg protein/ml of acid. The ampules were then evacuated to a pressure of less than 50 microns of mercury with alternate freezing and thawing until no further bubbling was seen when the sample was brought to room temperature. The ampules were sealed over a flame and placed in a 110°C oil bath.

Twenty-four hours later the ampules were removed from the bath, cooled, opened, and the HCl removed under reduced pressure at 40°C on an Evapo-mix apparatus (Buchler, Fort Lee), a process taking less than 15 min. The residue was then washed 3 times with distilled water and finally made up to the desired concentration with citrate buffer, pH 2.2. Amino acid analysis was performed on a Beckman 120 C amino acid analyser by the method of Spackman et al. (1958).

Results and Discussion

1. Physiochemical characterization of mouse serum albumin A and C monomer

As suggested by Peters (1970) a definition of albumin might specify a protein which (a) is soluble in half-saturated ammonium sulfate and in distilled water, (b) migrates on electrophoresis as the most abundant and one of the most acidic of the plasma proteins, (c)
exhibits a molecular weight of about 65,000 Daltons and sediments with a velocity of 4.5 Svedberg units in the ultracentrifuge, and (d) is free of carbohydrate by the usual tests. Additional criteria may include amino acid composition, and the ability to bind certain ligands such as bilirubin or fatty acid anions. Since these criteria were suggested for human albumin, a certain latitude is necessary when establishing homologies with 'albumins' of other species.

Proteins corresponding to human serum albumin on the basis of at least some of the above criteria have been observed in the plasma of some fish and amphibians (Engle et al., 1958; Wallace and Wilson, 1972), in reptiles (Masat and Dessauer, 1968), and in birds and mammals (Wallace and Wilson, 1972; Peters et al., 1958; Charlwood, 1961). Although mammalian serum albumins are among the most studied of all proteins, they still remain poorly understood. For instance, their function is still obscure (Peters, 1970) and amino acid sequence data are available on only one fragment each of rat (Bradshaw and Peters, 1969) and bovine (Shearer et al., 1967) albumin and 4 segments of human albumin (Bradshaw and Peters, 1969; Swaney and Klotz, 1970; Winter et al., 1972; Babin and Goos, 1973). Despite these drawbacks their physiochemical properties are well characterized, and provide a body of data with which other albumins may be compared.

In an effort to assess the similarity between mouse serum albumin and albumin of other species as well as to uncover any gross differences between the mouse A and C albumin allotypes, the extinction coefficient, sedimentation constant, molecular weight, free sulfhydryl content, amino acid composition and serological identity were
determined using the albumin A and albumin C (rechromatographed) 
monomer preparations (G-150, fraction II).

An extinction coefficient ($e_{1cm}^{1\%}$) of 5.2 was calculated for both 
the normal and variant monomer. This compared favourably with a value 
of 5.3 for human albumin (Cohn et al., 1947). The weighing of samples 
of the lyophilised protein dried at 105°C overnight produced a constant 
moisture loss of 3%. Protein weights were corrected for this in cal-
culating the extinction coefficient.

Sedimentation velocity analysis at a single concentration (0.7%) 
yielded a value of 4.5 S for the sedimentation constant ($S_{20,w}$) of the 
monomer of albumins A and C. This value compares favourably with 4.6 S 
for the human monomer (Oncley et al., 1947).

Figure 5 shows the results of polyacrylamide electrophoresis in 
sodium dodecyl sulphate. The known molecular weights of protein 
standards were plotted against their relative mobilities in the gel. 
A value of 67,000 Daltons was obtained for the molecular weight of the 
mouse A and C monomers as compared to 65,000 Daltons for human albumin 
monomer (Hughes, 1954). Although the heavier material isolated by 
Sephadex chromatography was also analysed by electrophoresis, it was 
impossible to determine the molecular weight of these species because 
of the absence of suitable standards and nonlinearity of the graph in 
this region.

Reaction of albumin A and C with an excess of 5, 5'-dithiobis-
(2-nitrobenzoic) acid (DTNB) gave a value of 0.6 free SH groups per 
molecule of albumin, consistent with values found for other albumins 
(Hughes, 1950; Janatova et al., 1968). However, in several
Figure 5

Molecular weight determination of mouse serum albumin A and albumin C monomer by SDS-polyacrylamide gel electrophoresis.

SDS-polyacrylamide electrophoresis (Weber and Osborn, 1969) was performed using ribonuclease A, MW 13,600; chymotrypsinogen A, MW 25,000; ovalbumin, MW 45,000; and bovine serum albumin MW 68,000 as standards. Bovine serum albumin was not included in the graph due to its coincidence with mouse serum albumin though its mobility was used in calculation of the regression line (Monroe 1860 programmable calculator).
determinations of the free thiol content of a single batch of albumin A monomer, a consistent value of 2.6 SH groups was obtained versus the expected value of 0.6 sulfhydryl groups per molecule found for the variant. Albumin apparently possesses a single free thiol group which participates in mixed disulfide formation in the plasma with small molecule thiols such as cysteine and to a lesser extent glutathione (King, 1961) resulting in the blockage of some 40% of these groups in the preparations. The high value obtained for the one preparation of albumin A could indicate the existence of a stable, partly denatured state exposing two free thiol groups otherwise buried or the reductive cleavage of a single disulfide bond. The problem was not investigated further.

The results of amino acid analysis of albumins A and C are shown in Tables 1 and 2. Cysteine was determined as S-carboxymethylcysteine and values for serine and threonine were corrected for destruction during hydrolysis using standards hydrolysed for 24 hr. Data are presented as residues per molecule of 67,000 Daltons and also as residues per 617 residues to facilitate comparison with the work of Popp (1966) and per 575 residues for comparison with human, bovine and rat albumin.

Alkylation time had been increased to 40 min because only after this time was alkylation as judged by the disappearance of cystine upon amino acid analysis complete. Alkylation had no affect on any of the other amino acids.

The amino acid analysis of the albumin A and C monomers leaves no doubt as to the identity of the molecule. The high values of
glutamic acid, lysine and histidine are characteristic of albumins as are also low values of glycine and tryptophan (Peters, 1970).

The results summarized in Table 1 indicate that albumins A and C differ by 4 residues of alanine, 3 of leucine and 2 of threonine. These are neutral amino acids and may reflect residue replacements resulting in no net change in charge on the molecule and thus undetectable by electrophoresis. As expected the analysis of the whole molecule gives no clue as to the residue replacement responsible for the mobility difference between albumins A and C.

A comparison of the analysis of albumin A from C3H mice and that of the electrophoretically identical (A) albumin from C57BL mice as reported by Popp et al. (1966) reveals that, though the molecules are obviously homologous some rather large differences exist in the values of several amino acids. Values for lysine, serine and alanine differ by from 15 to 25% in the two studies when data for both are expressed as residues per 617 residues. Agreement for histidine, arginine, glutamic acid, glycine, isoleucine and leucine are also rather poor while agreement for the other amino acids is within two residues. Perhaps a cause for these discrepancies lies in the fact that Popp's analysis was meant only to distinguish mouse serum albumin from "albumin esterase" or esterase-1 and was not intended as a rigorous analysis of the amino acid content of mouse albumin. In the present study the values for albumin A and C were calculated from 2 and 3 determinations respectively, of 24-hr hydrolyzates. In addition, the values reported here for mouse serum albumin closely resemble the values obtained for rat serum albumin (Peters, 1968) as would be
<table>
<thead>
<tr>
<th>Amino acid</th>
<th>albumin A (C3H)</th>
<th>albumin C</th>
<th>albumin A</th>
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<tr>
<td></td>
<td>Residues per Molecule</td>
<td>Integer</td>
<td>Residues per Molecule</td>
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<tr>
<td>Lysine</td>
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<td>16</td>
<td>16.4</td>
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<td>22.2</td>
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<tr>
<td>CM-Cysteine</td>
<td>30.6</td>
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<td>29.4</td>
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<td>31.7</td>
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<tr>
<td>Tryptophan</td>
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<td>1</td>
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</table>

Total          | 593             | 592       | 618       | 617     |

aAverage of 2 and 3 runs respectively for albumins A and C. Hydrolysed for 24 hr in vacuo at 110°C.

bCalculated assuming a molecular weight of 67,000 Daltons.

cCalculated assuming a molecular weight of 70,000 Daltons.

dCorrected for destruction during hydrolysis from standards hydrolysed for 24 hr.

eBased on response of 1 peptide to Ehrlich's reagent.

fPopp et al. (1961).

gS-carboxymethylcysteine.
Table 2. Comparison of amino acid analyses of mouse albumin A (C3H) and rat, bovine and human albumins.

<table>
<thead>
<tr>
<th>Species</th>
<th>Mouse (C3H)</th>
<th>Rat</th>
<th>Bovine</th>
<th>Human</th>
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<tr>
<td>Lysine</td>
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<td>51</td>
<td>58</td>
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<tr>
<td>Histidine</td>
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<td>14</td>
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<td>Arginine</td>
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<td>23</td>
<td>23</td>
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<tr>
<td>Aspartic acid</td>
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<td>51</td>
<td>54</td>
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</tr>
<tr>
<td>Threonine</td>
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</tr>
<tr>
<td>Serine</td>
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<td>27</td>
<td>23</td>
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<tr>
<td>Glutamic acid</td>
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<td>76</td>
<td>81</td>
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<tr>
<td>Proline</td>
<td>31</td>
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<td>28</td>
<td>25</td>
</tr>
<tr>
<td>Glycine</td>
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<tr>
<td>ε-Cystine</td>
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<td>Methionine</td>
<td>7</td>
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<td>1</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td><strong>574</strong></td>
<td><strong>575</strong></td>
<td><strong>575</strong></td>
<td><strong>577</strong></td>
</tr>
</tbody>
</table>

*From Table 1.
expected considering their phylogenetic proximity. It is extremely
doubtful that C3H albumin differs from that of C57BL mice to the extent
reflected in the two sets of data yet still conserves the total net
charge characteristic of normal albumin A.

One of the most sensitive methods to uncover structural dissimi-
larity between two molecules is double immunodiffusion against specific
antisera. Figure 6 shows the results of immunodiffusion of albumin A
and albumin C monomer developed with rabbit antisera to mouse serum
albumin. Complete coalescence of the precipitin arcs was observed
suggesting that the two allotypes are serologically identical
(Barrett, 1970).

Based on the above data mouse serum albumin appears to be similar
to the albumins of most other mammalian and avian species studied
(Peters, 1970). No consistent differences were found between albumins
A and C in sedimentation velocity, extinction coefficient, molecular
weight or sulfhydryl content. The possibility that these two mole-
cules differ in primary structure by more than the single residue res-
ponsible for the charge difference is suggested by the discrepancies
in amino acid content. This can only be confirmed however by indi-
vidual amino acid analysis of all peptides or by direct sequencing.

2. Characterization of electrophoretic trailing bands

Another similarity between mouse serum albumin and that of other
species is the presence of microheterogeneity within otherwise homo-
geous protein preparations (Petersen and Foster, 1965). This micro-
heterogeneity reveals itself in several forms in both bovine and
Figure 6

Double immunodiffusion of albumin A and albumin C Sephadex G-150 fraction II (monomer) and fraction I (albumin polymer) precipitated with mouse serum albumin antiserum.

Albumin A fraction I (a), albumin C fraction I (b), albumin A fraction II (c), albumin C fraction II (d). Center wells contain mouse serum albumin antiserum. Lyophilised protein (0.1%) dissolved in 0.1 M Tris-HCl buffer, pH 8.6 containing 0.2 M NaCl was present in each antigen well and antiserum was used undiluted. Slides were developed for 24 hr, washed and stained with amido black.
human serum albumin (Andersson, 1969). One component is mercap-
talum in which has one SH group; another fraction contains no SH
group but represents mixed disulfides with cysteine and glutathione
and a third includes albumin dimers, trimers, tetramers and higher
aggregates.

The first form can only be distinguished by ion-exchange chroma-
tography and its presence in preparations of mouse serum albumin has
not been demonstrated.

The value of less than one SH group per molecule of albumin ob-
tained upon reaction with DTNB probably reflects the presence of the
second type of microheterogeneity or mixed disulfide formation invol-
ving the free thiol group.

The third form of microheterogeneity, polymer formation, has al-
ready been suggested as the cause of the trailing bands on electro-
phoresis of ethanol-soluble material on starch gel at pH 8.6.

For investigations of amino acid replacements, the blockage of a
free thiol group would cause no problems as cysteine or glutathione
would be displaced upon reduction and alkylation prior to enzymatic
digestion but the presence of material that can be resolved as sepa-
rate components or starch gel or be isolated by molecular exclusion
chromatography leaves too much doubt as to the purity of the albumin
preparations. Though evidence has been presented in CHAPTER I to
suggest that the trailing bands and heavier components from C-150
chromatography are homologous and actually represent dimers, trimers
and higher polymers of mouse serum albumin, it was necessary to in-
vestigate this material in some detail in order to ascertain that,
in view of its reappearance upon storage in otherwise pure monomer preparations, it did not represent a complex of albumin with some other serum protein or non-albumin contaminant.

Hartley, Peterson and Sober (1962) demonstrated that in preparations of bovine serum albumin rich in dimer and higher polymers, treatment with 2-mercaptoethanol caused complete disappearance of the polymer material and at least 50% dissociation of the dimer. Similarly, treatment with 2-mercaptoethanol of purified frog albumin containing aggregates, restored most of the preparation to the monomeric form (Nagano et al., 1972). When samples of the ethanol-soluble mouse albumin A and albumin C material were treated with 0.015 M 2-mercaptoethanol then run in starch gels containing 2-mercaptoethanol at the same concentration, the more cathodal bands disappeared and a great reduction was seen in the staining intensity of the first trailing band. The monomer showed a concomitant increase in stain density.

More direct evidence of the polymeric nature of the trailing bands was obtained through methods suggested by Ressler (1973). Electrophoresis of albumin A and albumin C ethanol-soluble material and the heavier fractions as prepared by chromatography on G-150 in gels of 12, 14, 16 and 18% (w/v) starch revealed a pattern characteristic of a family of size isomers (Figure 7). The anodal mobility of all components progressively decreased as the gel concentration was increased.

Hedrick and Smith (1968) discuss three different systems which may be distinguished using this technique. The first, exemplified by bovine serum albumin describes molecules of the same charge but of
Figure 7

Effect of gel concentration on relative mobility at pH 8.6 of albumin monomer and trailing bands in starch gels.

Albumin A monomer (a), first (b), second (c) and third (d) trailing bands. All bands for albumin C showed a consistent, slightly reduced anodal mobility, otherwise patterns were identical.
different size designated as size isomers. Here a plot of the log relative mobility versus gel concentration will result in converging lines of progressively increasing negative slopes. Figure 7 (data for albumin A) shows that in a plot of this type where the mobilities of the bands are measured relative to bromophenol blue, the lines of the first (slope -0.252) and second trailing bands (slope -0.323) relative to the monomer (slope -0.198) converge at lower gel concentrations. The line of the third band is however almost parallel (slope -0.338) with that of the second trailing component.

In polyacrylamide these lines are expected to converge and ultimately intersect at a common point of low gel concentration if the isomers exhibit unit increases in molecular weight with respect to the monomer (see also Maurer, 1971). If this is seen to occur, a plot of the absolute value of the slope of these lines versus the expected molecular weight of the polymers will result in a straight line. This system as investigated using starch gel, adequately describes the trailing bands of mouse serum albumin. Figure 8 demonstrates that when the absolute value of the slopes of the lines for the first and second trailing bands are plotted against the calculated molecular weight of a mouse albumin dimer and trimer (assuming a molecular weight of 67,000 Daltons for the monomer) a straight line is obtained with a correlation coefficient of 0.999.

Why the lines of figure 7 do not intersect at a low gel concentration as observed in polyacrylamide is not known. Perhaps the relative mobility does not vary exponentially, as a function of starch concentration as it does in polyacrylamide. The degree of sieving
Figure 8

Plot of absolute values of the slopes obtained in Figure 7 against the calculated molecular weights for an albumin dimer, trimer and tetramer assuming a molecular weight of 67,000 Daltons for the monomer.

Regression line (Monroe 1860 calculator) calculated from the slopes of monomer, first and second trailing bands.

Regression line including third trailing band assuming it to represent a tetramer.
may be considerably less at higher concentrations (12% and above) than would be expected if the relationship were logarithmic.

The second system described by Hedrick and Smith (1968) concerns molecules of the same size but different charge. These "charge isomers" illustrated by the lactate dehydrogenase isozymes are characterized by parallel lines in the plot of relative mobility versus gel concentration. The behaviour of the third trailing band relative to the second is best described in this system as a charge isomer. In gels of increasing starch concentration, it migrates at a constant distance cathodal to the second trailing band and thus, when the relative mobilities are graphed, the slopes of the lines are almost equal. If this band is assumed to be a tetramer, the slope of its relative mobility when plotted in Figure 8 appears to bear no relationship to the other components of the series. If this band is considered to represent a modified trimer and is included in the regression, a higher correlation with the other 3 points is observed.

The nature of the third trailing band is not known nor was it investigated further, however it could be a contaminant of non-albumin origin or an albumin trimer of altered charge. If fitted to the regression line formed by the albumin monomer, dimer and trimer in Figure 8 its slope suggests a molecular weight of at least 200,000 Daltons.

The third system of Hedrick and Smith include those molecules that differ both in charge and size. Here the values of the relative mobilities of molecules of this type will cause the lines of Figure 7 to cross at gel concentrations in excess of that necessary for minimal
sioning effects. No relationships of this kind could be demonstrated for any components in these preparations.

Finally figure 6 shows that a specific antiserum directed against mouse serum albumin is capable of combining with the Sephadex G-150 fraction I material and that between the A and C preparations at least the primary components (dimer) show serological identity.

The behaviour of these components in the presence and absence of varying degrees of molecular sieving in starch, agar and dextran gels, in the presence of 2-mercaptoethanol, and their reaction with polyvalent antiserum and antiserum directed against mouse serum albumin alone provides good evidence that at least the first and second trailing bands represent the dimer and trimer of mouse serum albumin. The identity of the third trailing component which represents less than 1% of the alcohol-soluble material as judged visually on electrophoresis was not established.
CHAPTER IV

INVESTIGATION OF STRUCTURAL DIFFERENCES DISTINGUISHING

THE ALBUMIN A AND ALBUMIN C MONOMERS

Three approaches were taken to obtain further insight into the molecular differences between albumin A and albumin C monomers:

1. net charge differences as determined by iodoacetic acid,
2. peptide mapping after tryptic hydrolysis and
3. amino acid analysis of variant peptides.

Materials and Methods

1. Determination of the net charge difference between albumin A and albumin C at pH 5.4.

Serum from mice homozygous for albumin C was diluted 1:3 with 0.03 M borate gel buffer, pH 8.6. The albumin was assumed to be in a concentration of approximately 1% at this dilution. Lyophilised albumin C monomer was dissolved in the borate buffer at the same concentration.

On the basis of one free SH group per molecule of albumin, 0.14 M 2-mercaptoethanol diluted in gel buffer was added in an equal volume to 50 µl each of albumin C monomer and serum for a 50-fold molar excess of 2-mercaptoethanol. After reduction overnight at 4 C, 50 µl of 0.28 M iodoacetic acid dissolved in the same buffer and brought to pH 8.6 with concentrated NaOH was added to each preparation and the
reaction allowed to proceed for 48 hr at 4°C in the dark. Iodoacetate
was present in a two-fold molar excess over all free thiol groups.
The samples were then run in a 14% gel in a sodium acetate-ethylene-
diaminotetraacetic acid buffer, pH 5.4 (Biddle and Petras, 1957) with
untreated serum containing albumins A and C (diluted 1:9 with 0.03 M
borate buffer) and albumin A and albumin C monomers (0.3% in borate
buffer).

2. Tryptic hydrolysis

The S-carboxymethyl monomers of albumin A and albumin C were sub-
jected to enzymatic digestion at room temperature with trypsin
(Worthington, Freehold, 2X crystallized) according to the schedule
described by Winter et al. (1972).

Usually 25 mg of the alkylated derivative were digested at one
time. To a 1% solution of the protein in ammonium bicarbonate buf-
fer pH 8.6, 10 μl of a 1% solution of trypsin in 0.001 N HCl were added
at 0, 1 and 4 hours. The digestion was allowed to proceed for a further
16 hours. Each addition of trypsin to the albumin was in a ratio of
1:100 (w/w) resulting in a final trypsin concentration of 3%. At the
end of 20 hours the digest was frozen and lyophilised.

3. Peptide mapping

Descending chromatography and subsequent electrophoresis were
performed essentially as described by Bennett (1967).

Usually 1 to 2 mg of albumin A or albumin C digest in 25 μl of
0.1 M NH₄HCO₃ buffer, pH 8.6, were applied to a 18 x 22 1/2 in sheet
of Whatman 3MM filter paper serrated across the bottom edge. Phenol red was applied separately as a marker. The chromatography solvent, n-butanol:pyridine:acetic acid:water, (90:60:18:72) was prepared just before use and chromatography was allowed to proceed for 24 hr or until the phenol red had just left the bottom of the sheet. After drying overnight at room temperature the top 3 1/2 in of the paper was cut off and one 4 x 14 1/2 in strip of Whatman 3MM paper was stitched to each end. Electrophoresis was performed in a varsel cooled Michl tank with a 5000 V power supply at pH 6.5 (433 ml pyridine and 17 ml acetic acid diluted to 13:1 with water) or pH 3.1 (390 ml acetic acid and 35 ml pyridine diluted to 13:1) for 2 hr at 3000 V. After completion of the run the sheet was dried in a chromatography oven at 80 C for 30 min then dipped in 0.2% ninhydrin in 95% ethanol and developed for 20 min at 80 C.

4. Recovery and amino acid analysis of variant peptides

For preparative maps 3-5 mg of tryptic digest were applied to the paper and chromatography and electrophoresis carried out as described above. The papers were subjected to repeated spraying with 0.02% ninhydrin until the variant peptides could just be identified. Variant peptides from two preparative runs were cut out and eluted with 20% acetic acid. The acetic acid was evaporated and the residue washed 3 times with deionized distilled water. Peptides and their backgrounds were hydrolysed as described previously in CHAPTER II.

Amino acid analysis was performed on a single 0.63 cm I.D. column of DC-1A resin (Durram, Palo Alto) using the 'Pico Buffer System II
Results and Discussion

To date only two investigations giving evidence for an amino acid replacement in serum albumin have been reported. Gitlin et al. (1961) in their study of the more cathodally migrating human albumin variant, albumin B, suggested that on the basis of peptide maps of tryptic and chymotryptic digests of the normal (A) and variant (B) molecules and mobility differences between the molecules at different pH's, a glutamic or aspartic acid residue in albumin A had been replaced by a lysine residue in the abnormal albumin B. Winter et al. (1972) investigated two other albumin allotypes, Oliphant and Ann Arbor, exhibiting the albumin B mobility. They were able to demonstrate through amino acid sequencing that substitution of a lysine for a glutamic acid had occurred in both forms and concluded that these two variants and the albumin B studied by Gitlin were identical.

Evidence for an amino acid replacement in albumin C comes from the electrophoretic behaviour of this albumin at pH 5.4 after treatment with iodoacetic acid, and from the amino acid analysis of variant tryptic peptides eluted from peptide maps of the normal and abnormal molecules.

In order to determine the total charge difference between albumins A and C, the single free sulfhydryl group of the variant albumin, both in serum and as an isolated monomer, was alkylated with iodoacetic acid which would add an additional negatively charged carboxyl group. Figure 9 shows that treated albumin C in serum demonstrated an
Figure 9

Starch gel electrophoresis pH 5.4 of albumin C (monomer) fraction II and serum alone and alkylated with iodoacetate.

Albumin A (monomer) fraction II (a), albumin C (monomer) fraction II (b), albumin C (monomer) fraction II alkylated with iodoacetate (c), C3H serum (d), albumin C serum (e), albumin C serum alkylated with iodoacetate (f).
increased anodal mobility to a position midway between the untreated albumin monomers. This suggests that at this pH, two additional H\(^+\) ions are bound to albumin C resulting in the decreased anodal mobility. If the simplest hypothesis, involving a single residue change, is assumed, then the most likely substitutions include a lysine (pK\(_3\) 10.53) (White et al., 1960) an arginine (pK\(_3\) 12.48) or a histidine (pK\(_2\) 6.00) in albumin C for a carboxyl residue in albumin A.

Under the conditions of reduction and alkylation used, the treated albumin C monomer showed no increase in anodal mobility and remained indistinguishable from the untreated slow albumin monomer. The reason for this is not clear. The problem was however not investigated further.

To account more specifically for the observed charge differences, samples of purified A and C monomer were reduced, alkylated, submitted to enzymatic hydrolysis and the resulting peptides separated by chromatography and electrophoresis. Two chromatographic solvent systems and electrophoretic buffers varying between pH 3.1 and 6.5 were tested in an effort to generate peptide maps of a resolution adequate to uncover any peptide differences. At pH's above 4 an unresolved line of neutral peptides was always present and at pH 3.6, 2 darkly staining areas of several overlapping peptides made it impossible to determine if a change in this area had occurred.

Figure 10 shows peptide maps of the normal and variant monomer run at pH 3.1. In the map of the variant (C) albumin a single neutral peptide (alb-A peptide 1), present in the map of the normal (A) albumin, is seen to be absent while 3 new peptides (alb-C peptides 1, 2 and 3) which have no counterpart in the normal have appeared.
Since more than one peptide was found in peptide maps of albumin C than could be shown to be missing from maps of albumin A, an additional point of cleavage for trypsin and thus an additional arginine or lysine residue was expected in the abnormal molecule.

The amino acid analyses of the variant tryptic peptides eluted from peptide maps of albumins A and C are presented in Table 3. The one peptide (TP-A1) present in albumin A but missing from the maps of albumin C contained lysine, glutamic acid, proline, glycine, alanine, valine and leucine for a total of 18 residues assuming one residue of lysine. The three peptides which appeared to be consistently absent from maps of albumin A but present in those of albumin C, were recovered and analyzed for their constituent amino acids. When the amino acid composition of TP-C1 and TP-C2 were compared with TP-A1 they were found to contain no amino acids not found in the normal albumin peptide and, at least superficially, appeared to be derived from TP-A1. Both these peptides contained a single lysine residue as compared to one in the albumin A peptide but where the latter only showed three glutamic acid residues, TP-C1 and TP-C2 had a combined total of four. The number of each of the other residues of the two variant peptides, when combined, equalled the number of the corresponding residues in TP-A1. It appears then that a lysine residue has been substituted for a glutamic acid residue in albumin C creating a new cleavage point for trypsin.

The presence of TP-C3 remains unexplained. This peptide as isolated from the maps of albumin C appeared consistently and was the darkest staining of the three variant peptides. The possibility
Figure 10

Peptide maps of the soluble tryptic peptides of S-carboxymethyl albumin A and albumin C monomer.

Descending chromatography (vertical dimension) for 24 hr in n-butanol:pyridine:acetic acid: water (90:60:18:72) with subsequent electrophoresis at pH 3.1 for 2 hr at 3000 V. Albumin A peptide TP-A1 (1), albumin C peptides TP-C1 (1), TP-C2 (2), TP-C3 (3). Peptide maps were stained with 0.2% (w/v) ninhydrin in ethanol and dried at 90 °C for 20 min.
Table 3. Amino acid analysis of tryptic peptides

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Mole per mole peptide&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TP-A&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>4.2 (4)&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Proline</td>
<td>4.4 (4)</td>
</tr>
<tr>
<td>Glycine</td>
<td>1.9 (2)</td>
</tr>
<tr>
<td>Alanine</td>
<td>3.1 (3)</td>
</tr>
<tr>
<td>Valine</td>
<td>2.0 (2)</td>
</tr>
<tr>
<td>Leucine</td>
<td>2.3 (2)</td>
</tr>
<tr>
<td>Lysine</td>
<td>1.0 (1)</td>
</tr>
<tr>
<td>Arginine</td>
<td></td>
</tr>
</tbody>
</table>

| Total       | 18        | 8          | 10         | 8         |

<sup>a</sup>Assuming one residue of lysine or arginine.

<sup>b</sup>Integral value of moles amino acid per mole peptide.
exists that it is a peptide of marginal solubility and appears only intermittently and in reduced concentration. Furthermore, it has an amino acid composition qualitatively similar to the other peptides and no new amino acids are present other than arginine. A glutamic acid to lysine substitution may adequately explain the mobility difference between albumin A and albumin C, yet peptide TP-C3 may represent a second residue replacement within the molecule.

The substitution of a lysine for a glutamic acid residue in the albumin molecule is consistent with the replacement of a guanine by an adenine nucleotide in the albumin cistron of the DNA (Nirenberg et al., 1965). The triplets GAA and GAC are known to code for glutamic acid while AAA and AAG code for lysine.
CHAPTER V

GENERAL DISCUSSION

The physicochemical properties of mouse serum albumin differ little from those of most of the other mammalian albumins described in the past. All have sedimentation constants of between 4.0 and 5.0 S, molecular weights of between 65,000 and 70,000 Daltons and extinction coefficients of between 5.0 and 7.0. Furthermore, mouse albumins are similar to bovine and human albumins in the percentage of free thiol groups blocked and in the formation of polymers on isolation.

The evidence presented in this investigation suggests the replacement of a glutamic acid residue by a lysine residue in albumin C. Additional evidence for this should however also be obtained from the amino acid analysis of the variant peptides after chymotryptic hydrolysis and ultimately from the sequence of that region of the molecule believed to contain the replacement (Needleman, 1970). The presence of peptide TP-C3 in the peptide maps of albumin C suggests further deviation from the primary structure of albumin A. If this peptide represents a second amino acid substitution resulting in a change of charge, then a third substitution to an amino acid of opposite charge must be present elsewhere in the primary sequence as the glutamic acid to lysine replacement described here adequately accounts for the charge difference between albumin A and albumin C.

Due to the large size of the albumin molecule, a single polypeptide chain of 67,000 Daltons, many different peptides result from enzymatic digestion by even as specific a protease as trypsin. Amino acid
analysis suggests that a total of 73 peptides (sum of lysine and arginine residues plus one) would result from tryptic hydrolysis. The broader specificity of chymotrypsin involving the carboxyl groups of tyrosine, phenylalanine, tryptophan and leucine would produce at least 133 peptides. Digests of this complexity present formidable problems of peptide separation and characterization.

Further structural work on mouse serum albumin would benefit from a different approach making use of the selective cleavage of the peptide bond involving the carboxyl group of methionine by cyanogen bromide. This would result in 8 or 9 large peptides which could be isolated (Winter et al., 1972) and characterized separately (Babin and Goos, 1973). Peptide maps of enzymatic digests of these fragments would be considerably less complex than for the whole molecule.

The glutamic acid to lysine substitution is characteristic not only of the mouse variant described here but also of the two human variants investigated by Winter et al. (1972). The significance of this similarity cannot be ascertained from the present data. Human albumin B, however, believed also to represent a lysine for a glutamic acid replacement, was shown by Blumberg et al. (1968) to bind thyroxine more avidly than albumin A suggesting that a comparison of binding characteristics of mouse albumins A and C may uncover significant functional differences between the two molecules.

During this study the feasibility of detecting biochemical variants in a single animal, indistinguishable by conventional starch gel electrophoresis but resolvable by peptide mapping was considered. The prime considerations in such an approach include (1) the relative
abundance of the protein, (2) the ease of purification and (3) the resolving power of the peptide mapping technique. As has been demonstrated, serum albumin would be an ideal protein with which to begin such studies not only because it represents over 50% of the total plasma protein and can be obtained in a relatively pure state by trichloroacetic acid-ethanol extraction but also because, as pointed out by Wallace and Wilson (1972) the albumin structural gene is equivalent in size to the combined genes for cytochrome c, myoglobin, hemoglobin (α and β chains), and the fibrinopeptides A and B and is thus ideal for evolutionary studies.

Since blood or cells from only a single animal would be available for each analysis the limits of detection and resolution of the peptide mapping technique is critical. A system requiring extremely small amounts of protein yet able to resolve variant peptides identical in charge must be sought. A method satisfying these criteria would make use of a micro-scale peptide mapping procedure such as the one suggested by Watanabe and Yoshida (1971) in which thin-layer cellulose replaces filter paper sheets as the chromatographic-electrophoretic matrix and a control in the form of a radiolabeled normal protein of high specific activity is used as in the procedure of Carrel and Owen (1971).

Though the present study has been primarily biochemical with a heavy stress on methodology, "...biochemistry in relation to evolution must be taken in its biological context" (Watts, 1971). The field of population genetics has, within the last few years become preoccupied, almost exclusively, with the problem of protein polymorphism and protein evolution (Lewontin, 1973). The theorists (Kimura and Ohta, 1971; Crow
and Kimura, 1970) maintain that for there to be such variety of individual proteins at such a high proportion of loci within populations of organisms at least 95% of the molecular forms observed must be neutral and thus unaffected by natural selection. The students of natural populations (Petras et al., 1969; Selander and Yang, 1969) on the other hand point to the remarkable similarity and stability of allele frequencies from geographically widely separated locations as being suggestive of systematic selection pressures maintaining the alleles at their respective frequencies.

While biologists have been studying evolution at the population level, biochemists have accumulated much evidence for the broad variability in primary sequence tolerated by certain proteins while maintaining their characteristic functions over extreme phylogenetic distance (Margoliash et al., 1971). Though this is suggestive of widespread amino acid substitution undirected by natural selection, some rather remarkable adaptations of these and other proteins of related species imply subtle and purposeful alterations of sequence in response to the different environments encountered in niche exploitation (Schoffenielis, 1971b).

While the belief that all mutations that are fixed are adaptive, simply because an amino acid substitution must induce a conformational change, however small, in a protein thus altering its functional efficiency is quite unreasonable, so is the view that, with the exception of a few residues in the so-called "active-site" of an enzyme, any other position in the sequence may be filled by any amino acid that mutation might bring its way. It is doubtful, that with the exception of a few cases, the fate of an organism will depend on the occurrence of a single
In considering "the irksome problem of evolution" (Schoffeniels, 1971a), the answer, no doubt, will prove to lie somewhere between the two extremes. A few attempts to link the molecular data to population process (see Haigh and Smith, 1972) have been made but what is required are studies which combine both observations of the behaviour of a protein variant within a population and data on this same variant taken in its physiological context with regard to functional and structural alterations.

The potential utility of the mouse serum albumins A and C allotypes in an investigation of this type is obvious. An electrophoretically detectable variant of limited geographical distribution is available for introduction into populations otherwise monomorphic at the albumin locus and since this report has provided preliminary characterization of the variant and demonstrated the similarity between mouse albumin and albumin of other species, advantage may be taken of numerous studies to guide further comparative biochemical investigation.

The scope of such a study as is suggested here would be considerable but it may serve to clarify the biologist's concept of adaptation as a process beginning at the molecular level. It is hoped that the work reported here may make a contribution toward this goal.
CHAPTER VI

SUMMARY

1. All inbred strains and most natural populations of *Mus musculus*
   so far studied have only one type of albumin, albumin A. Five animals
   found in a single natural population near Windsor, Ontario, have not
   only albumin A but also a slower migrating electrophoretic component,
   albumin C. These mice were heterozygous at a locus controlling the
   molecular structure of albumin.

2. Albumin A was isolated from serum of C3H mice and albumin C
   from stocks derived from the wild animals by trichloroacetic acid-
   ethanol extraction with subsequent separation of the monomers and poly-
   mers on Sephadex G-150.

3. These preparations were judged free of non-albumin contamina-
   tion by starch gel electrophoresis, sedimentation velocity analysis and
   immuno-electrophoresis.

4. Both albumins A and C exhibited an extinction coefficient of
   5.2 at 280 nm, a sedimentation constant of 4.5 S, a molecular weight of
   67,000 Daltons and a free sulfhydryl content of 0.6 SH groups per mole-
   cule. They were similar in amino acid composition and showed complete
   serological identity. These properties are consistent with those of
   previously characterized albumins of other species.

5. The heavier material separated from the albumin monomer was
   found to form the trailing bands observed on electrophoresis of the
   acid-ethanol extracts. These components were shown to be albumin
   material by their reaction with mouse serum albumin antiserum.
Furthermore, the behaviour of the components in starch gels of increasing concentrations suggested that they were albumin polymers.

6. A net difference of two positive charges was found between albumins A and C on electrophoresis at pH 5.4. Peptide maps of the S-carboxymethyl monomers showed three variant peptides in the albumin C maps which after elution and amino acid analysis revealed that albumin A and albumin C differed by one amino acid residue which could explain the difference in mobility. The difference appeared to involve the replacement of a glutamic acid residue by a lysine in albumin C. The existence of a third abnormal peptide suggests further variability within the variant molecule.
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