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Cystic fibrosis
genetic disease
isoelectric focusing
$\alpha$-2-macroglobulin
protease inhibition

# Studies on Cystic Fibrosis Using Isoelectric Focusing. II. Demonstration of Deficient Proteolytic Cleavage of $\alpha_2$-Macroglobulin in Cystic Fibrosis Plasma

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### Extract

**A protein with an isoelectric point (pI) of 5.48 was found to be deficient in plasma from most cystic fibrosis (CF) homozygotes and obligate heterozygote carriers for CF as compared with normal control plasma. Purification of the protein with a pI of 5.48 from normal plasma was performed using ammonium sulfate precipitation, DEAE-cellulose and CM-cellulose chromatography, Sephadex G-200 gel filtration, starch block electrophoresis, and Sepharose 4B gel filtration. The purified protein migrated as a single band on polyacrylamide gel electrophoresis, and displayed a single arc on immunoelectrophoresis against polyvalent antiserum to whole human serum. Results from various techniques used in its characterization indicate that this protein is a fragment of $\alpha_2$-macroglobulin ($\alpha_2$M) which is derived from $\alpha_2$M by proteolytic cleavage of intact $\alpha_2$M subunits. Quantitation of $\alpha_2$M levels in plasma indicated no significant differences between levels of $\alpha_2$M in CF homozygote, obligate heterozygote carrier, or normal control plasma samples. Quantitation of arginine esterase activity in plasma treated with chloroform and ellagic acid indicated that both the total arginine esterase activity and that fraction of arginine esterase activity inhibited by soybean trypsin inhibitor (SBTI) were decreased in most CF homozygote and obligate heterozygote plasma samples relative to normal control values. The results of this study indicate that plasma samples from CF homozygotes and obligate heterozygote carriers for CF show deficient proteolytic cleavage of $\alpha_2$M as compared with normal control plasma, and suggest that a structural abnormality in $\alpha_2$M or a deficiency in plasma proteolytic activity may be responsible for this deficiency in proteolysis.**

### Speculation

**An abnormality in the binding affinity of $\alpha_2$M for plasma proteases may account for the presence of "factors" in CF homozygotes and obligate heterozygote carriers.**

Cystic fibrosis is a generalized metabolic disorder, resulting from an unknown genetic defect (33). It is generally thought to be transmitted as a single autosomal recessive trait (10, 33), although several authors have found evidence for genetic heterogeneity within the clinical entity known as CF (38, 53) and have suggested that the disease may actually be a group of closely related genetic abnormalities with similar pathologic consequences (53). Several reports have indicated that sera from patients with CF (CF homozygotes) and from obligate heterozygote carriers contain factors that may be characteristic of the disease and thus possibly related to the primary genetic defect in CF (7, 11, 50, 52, 53). Other factors are found in saliva and sweat from CF homozygotes (31) and have been produced from cultured cells derived from CF homozygotes and heterozygotes (4–6, 13). Partial characterization of the various CF factors suggests that they are closely related

structurally and functionally; generally they are small cationic proteins (molecular weight 1,000–10,000) that can bind or complex to immunoglobulin G (1, 2, 4, 5, 13, 50, 51).

Recently, Hirschhorn (22) and Conover *et al.* (13) presented evidence that the CF factor(s) may be a C3a (anaphylatoxin) or a kinin-like molecule (12), and they hypothesized that the primary defect in CF may be related to a quantitative or qualitative deficiency in anaphylatoxin inhibitor (AI), an enzyme similar to carboxypeptidase B (13). Although the possible identification of the CF factor(s) such as C3a has been questioned (1, 28), abnormalities in both complement-mediated cytotoxicity (51) and plasma kallikrein or arginine esterase activity (38, 39) have been reported for CF homozygotes and heterozygotes. In a previous study (52) we showed that sera from CF homozygotes and obligate heterozygote carriers are deficient in a protein of the $\beta$-globulin fraction, and we speculated that the deficiency might be responsible for the accumulation of CF factor in serum (48, 52). We now report available evidence that this protein is a proteolytic derivative (fragment) of $\alpha_2$-macroglobulin ($\alpha_2$M), a major inhibitor of kallikrein, plasmin, and similar plasma arginine esterases (20, 21, 24). The relationship of our findings to those of Conover *et al.* (13), Rao *et al.* (38, 39), and others suggests a possible mechanism for the pathophysiology of CF.

## MATERIALS AND METHODS

### CHEMICALS

All chemicals used were reagent grade unless otherwise indicated. Benzoyl-*D L*-arginine-*p*-nitroanilide (BAPNA), tosyl arginine methylester (TAME), trypsin three times crystallized (type III), soybean trypsin inhibitor (SBTI) (type I-s), ethylenediaminetetraacetic acid (EDTA), ellagic acid (practical grade), and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical Co., St. Louis, Mo. Dithiothreitol (DTT) was from Calbiochem, San Diego, Calif. Kaolin was from Allied Chemical Co., Morristown, N. J. DEAE-cellulose and CM-cellulose ion exchange resins (DE-52 and CM-52) were from Whatman. Sephadex G-200 and Sepharose 4B were from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Purified $\alpha_2$-macroglobulin (1 mg) used for comparative purposes was a gift from Mrs. Helga Johnson.

### ANTISERA

Antisera to human $\alpha_2$-macroglobulin, plasminogen, and haptoglobin were obtained from Behring Diagnostics, Woodbury, N. Y. Antisera to human albumin, IgM, C3, and whole serum were from Meloy Laboratories, Springfield, Va.

### PLASMA

Venous blood from normal individuals and from CF patients and parents (obligate heterozygote carriers) was collected in plastic syringes or plastic transfer packs and mixed into 1:9 volume of 3.8% (w/v) sodium citrate in polypropylene tubes. Plasma was separated by centrifugation at 2,000 × *g* for 10 min at 4° (20) and frozen in aliquots in polypropylene at −70°. The plasma was thawed only once for use.

Informed consent was obtained from each subject (or in the case of minors, from their parents) before collection of the plasma.

### ELECTROPHORESIS

Immunoelectrophoresis (IEP), counterimmunoelectrophoresis (CIE), and double immunodiffusion were performed by established methods (37). Quantitation of $\alpha_2$M in whole blood and in various fractions (37) was performed by radial immunodiffusion (54).

For analytic acrylamide gel electrophoresis, an apparatus similar in construction to that described by Raymond (42), was used, with a gel concentration of 4% and with Tris·HCl glycine buffer, pH 8.9 (14).

Starch block electrophoresis was performed by slight modification of the methods of Kunkel and Trautman (25) and McConnell (34), with the electrophoresis conducted at 300 V for 14 hr using 0.1 M phosphate-0.15 M NaCl buffer, pH 7.5, and samples introduced into a block 1.5 × 10 × 47 cm, 10 cm from the cathodal end. At the completion of a run, each 1-cm section was eluted three times with 20 ml 0.01 M phosphate-0.15 M NaCl buffer, pH 8.0. After clarification by centrifugation (2,000 × g, 15 min, 4°) the eluates were concentrated in an Amicon ultrafiltration cell equipped with an XM50 membrane (55).

### ELECTROFOCUSING

Isoelectric focusing in thin layer polyacrylamide gels with 4 M urea added was performed as described previously (52), except that only gradients of pH 3–8 were used. A linear gradient (Fig. 1) was constructed by mixing pH 2.5–4, 3.5–5, 4–6, and 5–8 Ampholine carrier ampholytes (56) to obtain a final concentration of 2.1% (w/v). Electrofocusing was conducted at 500 V for 18 hr. Isoelectric focusing was used throughout the investigation to follow the isolations of the $\alpha_2$M subunits and fragments with pI's at and near pH 5.48, to analyze whole plasma samples subjected to various treatments, and to assess the conversion of $\alpha_2$M to its subunits (mol wt 185,000) and fragments (mol wt 85,000) (21).

### ENZYMATIC REACTIONS

For measurement of total plasma arginine esterase activity, Hageman factor was activated with chloroform and 0.05 mM ellagic acid in 0.1 M phosphate-0.15 M NaCl buffer (phosphate saline), pH 7.5, exactly as described by Rao *al et.* (39). Arginine esterase activity was assayed with TAME as substrate (0.015 M) in phosphate saline (pH 7.6) according to the methods of Siegelman *et al.* (45). Activity was measured in the presence and absence of SBTI at 400 $\mu$g/ml (38, 39) in phosphate saline, pH 7.6.

For isoelectric focusing, Hageman factor was activated either by incubation of whole plasma in glass at 4° for 2 hr (32) or by treatment with kaolin (10 mg/ml plasma, 15 min, 25°) (9, 20) or ellagic acid (without prior treatment with chloroform).

In experiments employing trypsin to convert purified $\alpha_2$M to its proteolytic derivatives (21), the specific activity of trypsin preparations was experimentally determined by titration with SBTI following the methods of Ganrot (16, 17) and Harpel (21) with BAPNA as substrate. Esterolytic activity of trypsin preparations was determined by the method of Siegelman *et al.* (45) with trypsin in 0.05 M $CaCl_2$ and TAME as substrate.

The functional capacities of different $\alpha_2$M preparations were assessed by determining the esterolytic activity associated with $\alpha_2$M with and without trypsin added at different molar ratios (21) before and after the addition of SBTI (16, 18, 21). Trypsin was

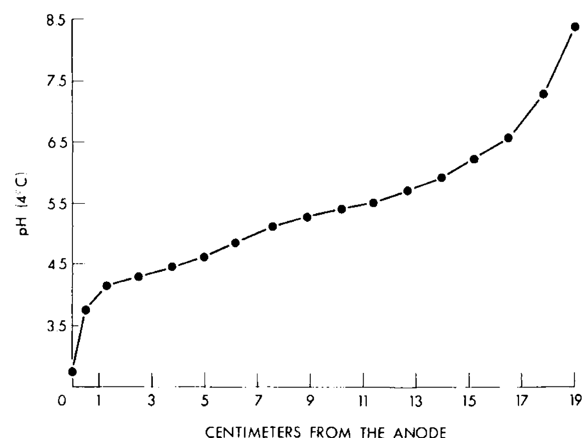


Fig. 1. Result of pH measurement at 4° from anode to cathode on the surface of a 5% polyacrylamide gel containing 2.1% (w/v) Ampholine carrier ampholytes, pH 3–8, 4 M urea.

incubated with $\alpha_2$M for 30 min at 37° in phosphate saline (pH 8.0) before the addition of SBTI (400 $\mu$g/ml).

The functional states of purified $\alpha_2$M preparations of $\alpha_2$M treated with trypsin were also determined by SDS-polyacrylamide gel electrophoresis, with or without prior reduction with DTT, as described by Harpel (21), except that $\alpha_2$M and $\alpha_2$M-trypsin mixtures were incubated in phosphate saline (pH 8.0) and electrophoresis was conducted in a 5% slab gel using Tris-acetate-EDTA buffer, pH 7.4 (15) and an apparatus similar to that of Raymond (42). Molecular weight markers included human serum albumin in reduced form (68,000) and purified $\alpha_2$M in reduced (approximately 85,000) and nonreduced (approximately 340,000) form (21).

### PURIFICATION OF $\alpha_2$M AND $\alpha_2$M FRAGMENTS OBSERVED BY ISOELECTRIC FOCUSING ($\alpha_2M_f$)

All isolation procedures were performed at 4° using plastic or siliconized labware. Protein concentrations of preparations were determined by the method of Lowry *et al.* (30) with bovine serum albumin as standard. Protein concentrations of column eluates were estimated by recording their absorbance at 280 nm. Fractions were concentrated in Amicon stirred ultrafiltration cells of 50 or 10 ml capacity equipped with an XM50 membrane (55). Protein compositions of individual fractions or purified products were assessed by IEP, CIE, and double immunodiffusion with specific antisera where indicated. All isolation procedures were repeated a minimum of three times.

### ISOLATION OF $\alpha_2$M FRAGMENTS OBSERVABLE BY ISOELECTRIC FOCUSING ($\alpha_2M_f$)

Chilled plasma (100–200 ml) was brought to 25% saturation by the addition of saturated ammonium sulfate (100%, 4°) while stirring. After 30 min the mixture was centrifuged (12,000 × *g*, 30 min); the supernatant was then removed and brought to 50% saturation with saturated ammonium sulfate, stirred for 30 min, and the precipitate was harvested by centrifugation (12,000 × *g*, 30 min). The precipitate was resuspended in 0.1 M phosphate-0.15 M NaCl buffer, pH 8.0, and dialyzed exhaustively against 0.01 M phosphate buffer, pH 8.0, in preparation for chromatography. A concentrated preparation of the 50% precipitated proteins (100 mg/ml) was then applied to a DEAE-cellulose column (3.0 × 45 cm) previously equilibrated with 0.01 M phosphate, pH 8.0. Fractionation was performed with a continuous sodium chloride gradient (0.00–0.2 M NaCl) or a discontinuous gradient (in consecutive steps) by elution with 300 ml volume of 0.01 M phosphate plus either 0.00 M, 0.05 M, 0.1 M, or 0.2 M NaCl.

Fractions containing $\alpha_2M_f$ were then dialyzed against 0.02 M sodium acetate-0.05 M NaCl buffer, pH 5.0, concentrated to 30 mg/ml, and fractionated on a CM-cellulose column (2.2 × 40 cm) previously equilibrated with 0.02 M sodium acetate buffer, pH 5.0. After washing the column with 250 ml of 0.02 M sodium acetate-0.05 M NaCl buffer (pH 5.0), the fraction that contained $\alpha_2M_f$ was eluted with 0.02 M sodium acetate-0.15 M NaCl, pH 5.0. The 0.15 M NaCl eluate was concentrated to 8 mg/ml and applied as separate 3.0-ml aliquots to a calibrated Sephadex G-200 column. Elution was performed in 0.01 M phosphate-0.15 M NaCl buffer, pH 8.0. Individual fractions containing $\alpha_2M_f$ from separate G-200 colums were pooled, concentrated, and subjected to starch block electrophoresis. Fractions containing $\alpha_2M_f$ obtained from starch block electrophoresis were further purified over a calibrated Sepharose 4B column (2.2 × 100 cm) using 0.01 M phosphate-0.15 M NaCl, pH 8.0. Final preparations of $\alpha_2M_f$ had average TAME esterase activity levels of 1.2 $\mu$mol TAME hydrolyzed/min/mg $\alpha_2M_f$ (21, 45). The Sephadex G-200 and Sepharose 4B columns were calibrated with blue dextran (mol wt 2,000,000; Pharmacia), IgM (mol wt 820,000), IgG (mol wt 150,000), human serum albumin (mol wt 68,000), and chymotrypsinogen A (mol wt 25,000; Pharmacia) as indicated.

### ISOLATION OF $\alpha_2$M

Purified $\alpha_2$M was obtained by the following procedure, adapted from Harpel (21) and McConnell (34). Whole plasma was mixed with SBTI (0.2 mg/ml) and EDTA (10 mM) and dialyzed against 0.01 M phosphate buffer, pH 8.0, containing 10 mM EDTA (phosphate EDTA). The whole plasma was then fractionated by DEAE-cellulose chromatography as described for $\alpha_2M_f$, except that the column was pre-equilibrated with phosphate EDTA and washed with 300 ml phosphate EDTA and 0.05 M NaCl immediately after application of the plasma. The $\alpha_2$M fraction was then eluted with 300 ml 0.1 M NaCl, concentrated, and fractionated by Sephadex G-200 gel filtration, starch block electrophoresis, and Sepharose 4B gel filtration. Preparations of $\alpha_2$M found to be contaminated with haptoglobin or IgM were rerun on starch block electrophoresis and/or Sepharose 4B gel filtration. Purified preparations of $\alpha_2$M gave a single arc when analyzed by immunoelectrophoresis against potent polyvalent goat or rabbit anti-human serum. These preparations had low levels of spontaneous TAME esterase activity, averaging 0.10 $\mu$mol TAME hydrolyzed/min/mg $\alpha_2$M (45).

## RESULTS

### ANALYSIS OF WHOLE PLASMA BY ISOELECTRIC FOCUSING

Twenty-five-microliter aliquots of freshly thawed plasma from CF homozygotes, obligate heterozygote carriers, and normal controls were analyzed by isoelectric focusing. Before activation, a protein band with a pI of 5.48, which was observed previously in normal whole serum, was absent from all plasma samples. After activation in glass, however, a pattern qualitatively similar to that observed for serum was found in all normal controls but not in the majority of CF homozygote samples (Fig. 2, Table 1). Typical protein banding patterns for three normal and three CF homozygote plasma samples are shown in Figure 3. (The centimeter scale in Figures 2–4 correlates with the pH gradient in Figure 1.) Most activated CF plasma samples lacked band 1 (Table 1), and the majority were deficient in a second protein band (band 2) just

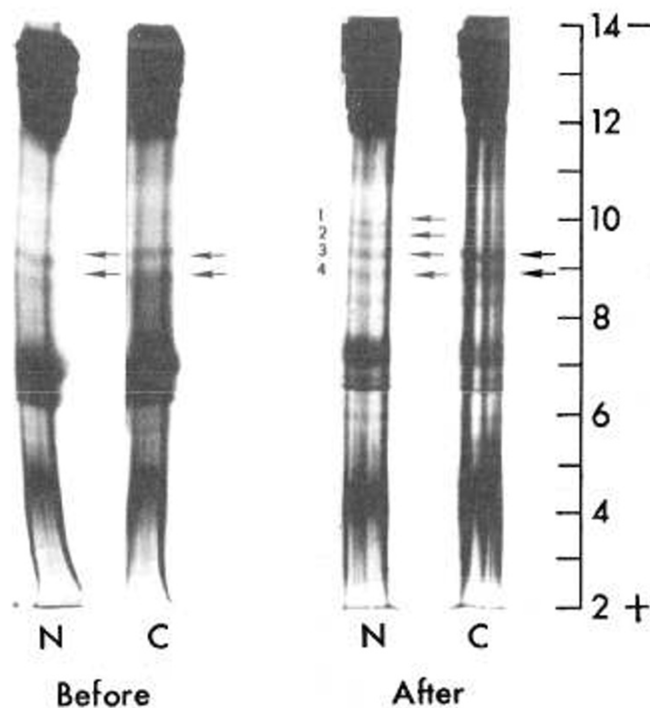


Fig. 2. Results of isoelectric focusing of 25 $\mu$l whole plasma from a CF homozygote (*C*) and normal control (*N*) individual before and after activation of the plasma by glass. A centimeter scale shows the distance from the anode.

Table 1. *Relationship between $\alpha_2$-macroglobulin level, arginine esterase activity, and detection of $\alpha_2$-macroglobulin fragments in activated plasma*[1]

| Sample | Age, yr | Sex | $\alpha_2$-Macroglobulin level,[2] mg/100 ml | Arginine esterase activity[3] Total activity | SBTI inhibited activity | SBTI-resistant activity | $\alpha_2M_F$ Band[4] 1 | 2 | Band 5 |
|---|---|---|---|---|---|---|---|---|---|
| Cystic fibrosis homozygote | | | | | | | | | |
| 1. C7422 | 25 | M | 166 | 17.45 | 9.42 | 8.03 | − | − | N[6] |
| 2. C13718 | 19 | F | 191 | 18.69 | 4.12 | 14.57 | +[5] | + | S |
| 3. C192112 | 5 | M | 260 | 24.65 | 13.06 | 11.59 | − | + | N |
| 4. C18198 | 24 | M | 151 | 18.78 | 12.72 | 6.06 | − | − | N |
| 5. C1381 | 15 | F | 170 | 22.12 | 8.46 | 13.66 | ± | − | S |
| 6. C71121 | 20 | M | 200 | 21.65 | 17.52 | 4.13 | − | − | N |
| 7. C425 | 10 | M | 287 | 27.06 | 15.59 | 11.47 | − | ± | N |
| 8. C20215 | 14 | M | 240 | 15.92 | 4.57 | 11.33 | ± | + | S |
| 9. C13131 | 8 | F | 205 | 18.14 | 11.49 | 6.65 | − | − | N |
| 10. C1381A | 11 | F | 195 | 13.76 | 8.49 | 5.27 | − | − | N |
| 11. C18218 | 12 | M | 150 | 15.50 | 8.31 | 7.19 | − | − | N |
| 12. C2045 | 12 | F | 140 | 17.62 | 9.20 | 8.42 | − | − | N |
| Mean ± SD | | 6M | | | | | 1+ | 3+ | 3S, |
| and totals | 15 ± 6 | 6F | 196 ± 46 | 19.27 ± 3.90 | 10.25 ± 4.03 | 9.03 ± 3.40 | 2± | 1± | 9N |
| | | | | | | | 9− | 8− | |
| Heterozygote carrier | | | | | | | | | |
| 1. H4225 | 28 | F | 153 | 22.92 | 12.92 | 10.00 | − | + | N |
| 2. H13718 | 57 | F | 127 | 25.59 | 15.19 | 10.49 | + | + | N |
| 3. H131915 | 23 | F | 176 | 17.99 | 8.00 | 9.99 | ± | + | N |
| 4. H481 | 36 | F | 151 | 18.38 | 11.52 | 6.86 | ± | + | N |
| 5. H13131 | 40 | M | 102 | 31.13 | 18.79 | 12.34 | + | ± | N |
| 6. H1279 | 23 | F | 223 | 33.66 | 31.46 | 2.20 | + | + | N |
| 7. H122112 | 24 | F | 110 | 28.52 | 21.22 | 7.30 | + | + | N |
| 8. H1681 | 37 | F | 176 | 20.25 | 11.25 | 9.00 | ± | ± | N |
| 9. H245 | 43 | M | 145 | 16.47 | 13.60 | 2.87 | ± | + | N |
| 10. H16198 | 57 | F | 87 | 25.00 | 17.43 | 7.57 | + | + | N |
| 11. H481A | 40 | M | 110 | 28.71 | 22.70 | 6.01 | + | + | N |
| Mean ± SD | | 3M | | | | | 6+ | 9+ | |
| and totals | 37 ± 12 | 8F | 142 ± 40 | 24.78 ± 5.56 | 16.73 ± 6.61 | 7.67 ± 3.15 | 4± | 2± | 11N |
| | | | | | | | 1− | | |
| Normal controls | | | | | | | | | |
| 1. N12218 | 24 | F | 191 | 45.70 | 28.30 | 17.40 | ± | + | N |
| 2. N18181 | 29 | M | 94.2 | 42.17 | 26.08 | 16.09 | + | + | N |
| 3. N4715 | 25 | M | 139 | 34.42 | 25.78 | 8.64 | + | + | N |
| 4. N21110 | 21 | F | 161 | 46.20 | 30.50 | 15.70 | + | + | N |
| 5. N5112 | 29 | F | 163 | 39.10 | 25.00 | 14.10 | + | + | N |
| 6. N7239 | 26 | M | 81 | 27.15 | 20.02 | 7.13 | + | + | N |
| 7. N131920 | 28 | F | 191 | 36.52 | 33.62 | 2.90 | + | + | N |
| 8. N7125 | 26 | M | 149 | 42.84 | 36.05 | 6.79 | + | + | N |
| 9. N10193 | 32 | F | 129 | 38.39 | 23.62 | 14.77 | + | + | N |
| 10. N10101 | 28 | F | 176 | 30.98 | 18.89 | 12.09 | + | + | N |
| 11. N3133 | 23 | F | 171 | 33.71 | 22.47 | 11.24 | + | + | N |
| Mean ± SD | 26 ± 3 | 4M | 150 ± 36 | 37.93 ± 6.08 | 26.39 ± 5.37 | 11.53 ± 4.63 | 10+ | | |
| and totals | | 7F | | | | | 1± | 11+ | 11N |

[1] M: Male; F: Female; SBTI: soybean trypsin inhibitor; $\alpha_2M_F$: fragment of $\alpha_2$-macroglobulin observed by isoelectric focusing.
[2] Measured by single radial immunodiffusion: values expressed to the nearest whole unit.
[3] Micromoles of tosyl arginine methyl ester hydrolyzed per hr per ml of plasma.
[4] Bands 1 and 2 as in Figures 3 and 4.
[5] +, ±, −: bands 1 and 2 strong, faint and not present, respectively, when 25 μl of activated plasma was analyzed.
[6] Intensity of band 5 on isoelectric focusing. S: strong; N: normal.

below band 1 (Figs. 3 and 4, Table 1). CF plasma samples that were quantitatively normal for bands 1 and 2 (+ or ±), displayed a strong band (band 5) (Table 1, Fig. 3) with a pI of approximately 4.7 and an additional band just above it (arrow in Fig. 3), not found in normal controls. Obligate heterozygote carriers showed bands qualitatively similar to the normal controls, but band 1 was generally weaker (Table 1).

Bands 1 and 2 could be demonstrated only in glass-activated plasma. Since Hageman factor is known to be activated by glass and then in turn is involved in the activation of several other plasma proenzymes (*i.e.*, plasminogen, plasminogen proactivator, prekallikrein) (24, 32, 40), we used two other known activators of Hageman factor to determine whether bands 1 and 2 could be demonstrated in the CF samples that were negative after glass

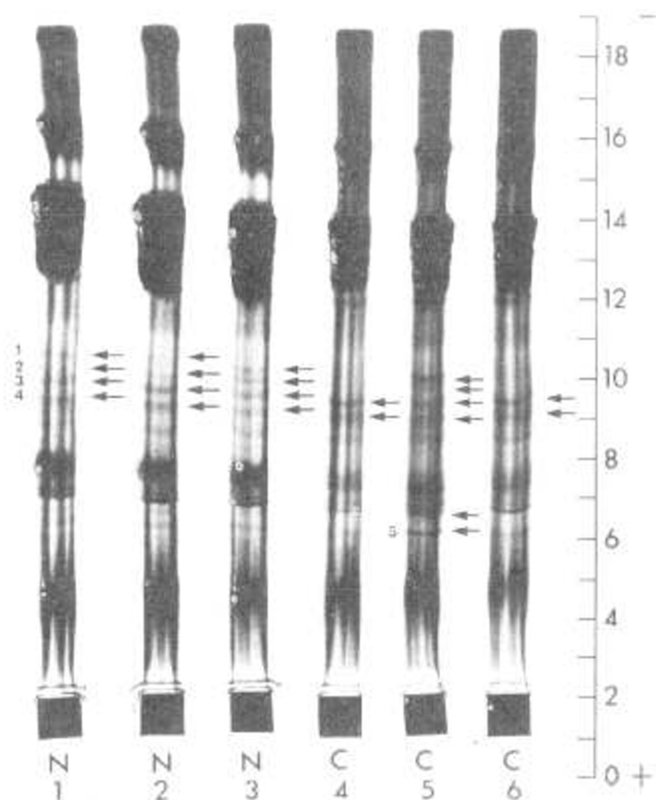


Fig. 3. Results of isoelectric focusing of 25 $\mu$l glass activated plasma from three normal control (*N*) and three cystic fibrosis homozygote (*C*) individuals. A centimeter scale shows the distance from the anode.

activation. Incubation with kaolin or ellagic acid gave results nearly identical to those for CF and normal samples after glass activation.

### ISOLATION OF $\alpha_2$M AND ITS PROTEOLYTIC DERIVATIVES

Protein band 1 (Fig. 4, *sample A*) was selectively concentrated from freshly thawed plasma by fractionating 100 ml of normal plasma with 25% and then 50% $(NH_4)_2SO_4$. The protein was shown to be in the 50% $(NH_4)_2SO_4$ precipitate by electrofocusing (Fig. 4, *sample B*). Further fractionation of 1.0 g of the precipitated protein was carried out on a DEAE-cellulose column with a gradient of 0.00 to 0.2 M NaCl at constant pH (Fig. 5*A*). Subsequent analysis of the 11 fractions from the eluate showed that band 1 eluted in fractions VI and VII, corresponding to a NaCl concentration of 0.060 to 0.082 M. The results of isoelectric focusing of fraction VII are shown in Fig. 4, *sample C*. Results from DEAE-cellulose chromatography with discontinuous gradient elution showed that the 0.1 M NaCl eluate was the only fraction that contained this protein.

Further purification was accomplished by CM-cellulose chromatography using discontinuous gradient elution with NaCl at constant pH. The protein band with a pI at 5.5 was found in the 0.15 M NaCl eluate (Fig. 4, *sample D*). Fractionation of the 0.15 M NaCl CM-cellulose eluate by Sephadex G-200 gel filtration yielded three peaks, corresponding to fractions with molecular weights greater than 250,000 (peak I), between 250,000 and 150,000 (peak II), and less than 150,000 (peak III) (Fig. 5*B*). Analysis of each peak by isoelectric focusing showed the band 1 protein in peak II (Fig. 4, *sample E*). Peak I contained approximately 3 times as much protein as peak II but showed no bands when analyzed by isoelectric focusing using 100 $\mu$l of a 6 mg/ml solution. Analysis of Sephadex G-200 peaks I and II by immunoelectrophoresis, however, indicated that they contained almost the same proteins. Peak I was composed predominantly of $\alpha_2$M, haptoglobin, and IgM with trace amounts of C3; peak II contained $\alpha_2$M, C3, and traces of haptoglobin and albumin (apparently in polymeric form).

Final purification was accomplished by starch block electrophoresis and Sepharose 4B gel filtration. The fraction containing protein band 1 was found in the fraction that eluted just after IgM (mol wt 820,000) (Fig. 5*C*). The final product displayed a single arc on immunoelectrophoresis against potent polyvalent rabbit or goat anti-human serum (Fig. 6*A*) and migrated as a single band on acrylamide gel electrophoresis at pH 8.9 (Fig. 6*B*). In tests against antisera to haptoglobin, IgM, C3, albumin, and $\alpha_2$M, only anti-$\alpha_2$M showed a precipitation line when analyzed by immunoelectrophoresis, CIE, or double immunodiffusion. Isoelectric focusing, however, showed four closely spaced bands (Fig. 4, *sample F*), the uppermost band having a pI corresponding to band 1 of the whole activated plasma (Fig. 4, *sample A*).

### ANALYSIS OF $\alpha_2$M AND $\alpha_2M_f$ BY SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Since $\alpha_2$M was found to represent bands 1 and 2, we next sought to explain why $\alpha_2$M in peak I from Sephadex G-200 gel filtration (Fig. 5*B*) would not focus in a 5% thin layer polyacrylamide gel, whereas $\alpha_2$M in peak II did. (The $\alpha_2$M derivatives that could be focused by IEF are designated as $\alpha_2M_f$.)

Analysis of purified $\alpha_2$M and $\alpha_2M_f$ on SDS-polyacrylamide gel electrophoresis without reduction revealed no consistent differences between the two preparations (Fig. 7). However, when the preparations were reduced with DTT before electrophoresis, $\alpha_2$M showed one major band (average mol wt of 185,000), whereas $\alpha_2M_f$ showed two bands (mol wt 185,000 and 85,000). This suggests that the $\alpha_2M_f$ preparation contains not only intact $\alpha_2$M subunits, but also subunit derivatives, which apparently result from proteolytic cleavage during isolation, as a result of the interaction

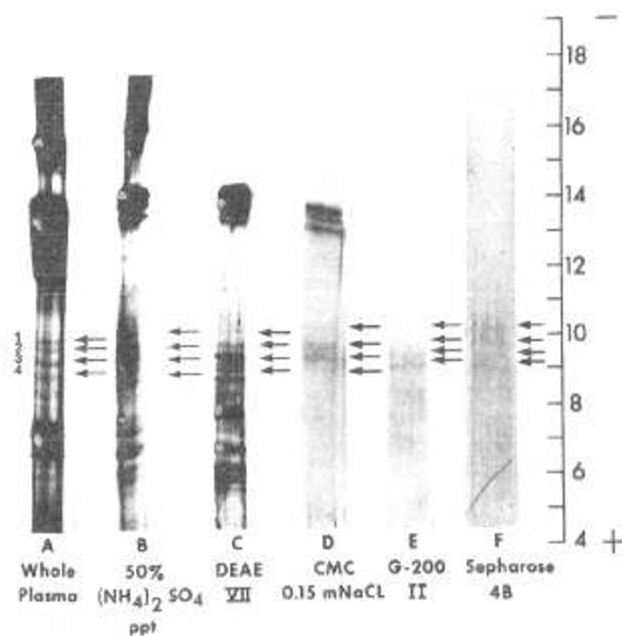


Fig. 4. Isolation of fragment of $\alpha_2$-macroglobulin ($\alpha_2M_f$) as shown by isoelectric focusing. *A:* 25 $\mu$l whole activated normal plasma; *B:* 25 $\mu$l 50% $(NH_4)_2SO_4$-precipitable proteins (50 mg/ml); *C:* 25 $\mu$l fraction VII from DEAE-cellulose chromatography (50 mg/ml) (see Fig. 5*A*); *D:* 50 $\mu$l 0.15 M NaCl eluate from CM-cellulose (*CMC*) chromatography (30 mg/ml); *E:* 50 $\mu$l peak II from Sephadex G-200 gel filtration (30 mg/ml) (see Fig. 5*B*); *F:* 50 $\mu$l Sepharose 4B eluate (6 mg/ml). The major bands found in purified preparations of $\alpha_2M_f$ are indicated by arrows. A centimeter scale shows the distance from the anode.

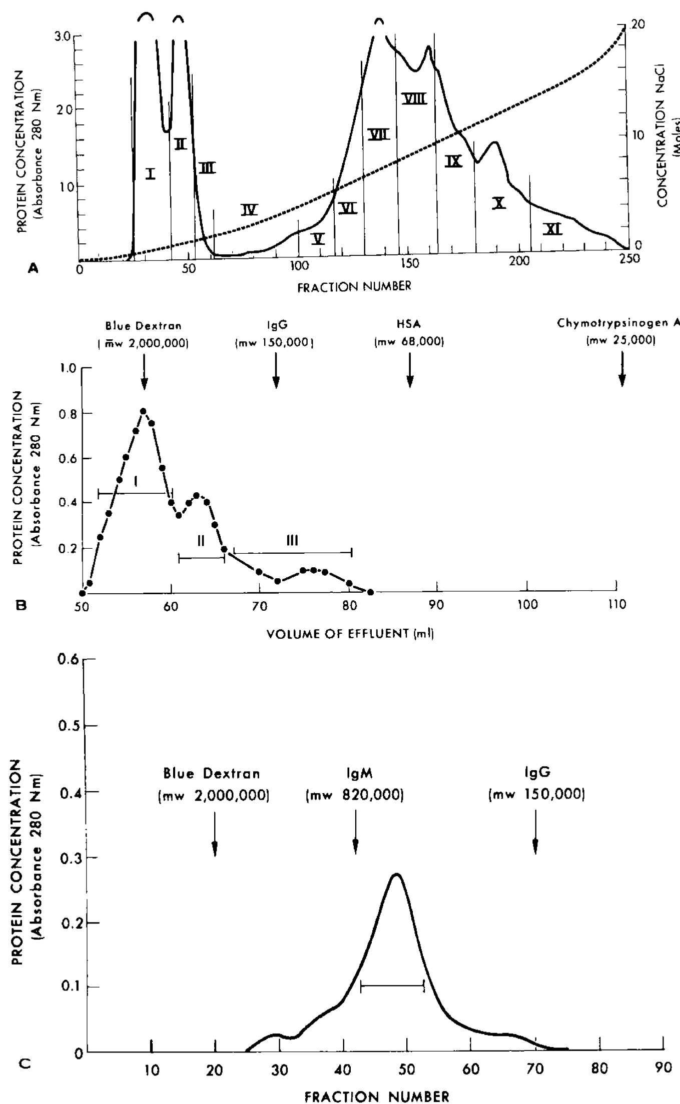


Fig. 5. *A:* DEAE-cellulose chromatography of 1,000 mg $(NH_4)_2SO_4$-precipitable proteins. Chromatography was performed on a column, 3.5 × 43 cm, in 0.01 M phosphate buffer, pH 8.0, with a continuous NaCl gradient. The flow rate was 25 ml/hr. Four-milliliter fractions were collected. ——: protein concentration (absorbance at 280 nm); - - -: NaCl concentration (moles). *B:* Sephadex G-200 gel filtration of 24 mg, CM-cellulose 0.15 M eluate. Elution was performed on a calibrated column, 2.2 × 100 cm, in 0.01 M phosphate buffer-0.15 M NaCl, pH 8.0, at a flow rate of 6 ml/hr. The molecular weight markers used for calibration of the column are shown. *HSA:* human serum albumin. *C:* Sepharose 4B gel filtration of 15 mg protein obtained after starch block electrophoresis. Elution was performed on a calibrated column, 2.2 × 100 cm, in 0.01 M phosphate buffer-0.15 M NaCl, pH 8.0, at a flow rate of 6 ml/hr. The molecular weight markers used for calibration of the column are shown. Five-milliliter fractions were collected. The position of elution of $\alpha_2$-macroglobulin or $\alpha_2$-macroglobulin fragment is indicated by the bar.

of $\alpha_2$M with one or more of its proteolytically (esterolytically) active enzyme substrates found in plasma.

Additional evidence that $\alpha_2M_f$ is a mixture of intact $\alpha_2$M and $\alpha_2$M that has been proteolytically cleaved was obtained by reacting trypsin with purified $\alpha_2$M at different molar ratios (21). Electrophoresis of reduced samples (Fig. 7) showed that treatment of $\alpha_2$M with trypsin at increasing molar ratios of trypsin to $\alpha_2$M caused a progressive conversion of intact $\alpha_2$M subunits (mol wt 185,000) to their proteolytically cleaved derivatives (mol wt 85,000) and revealed a pattern nearly identical with that shown for $\alpha_2M_f$ without prior trypsin treatment (Fig. 7).

## TRYPSIN BINDING CAPACITY OF $\alpha_2$M AND $\alpha_2M_f$

Further evidence was found when the functional capacities of $\alpha_2$M and $\alpha_2M_f$ preparations (1 mg/ml) were assessed by their ability to protect the esterolytic activity of trypsin from inhibition by SBTI (400 µg/ml). The results are shown in Figure 8, with a curve depicting the esterolytic activity of trypsin without prior treatment for comparison. Addition of $\alpha_2$M to trypsin reduced the specific activity of the enzyme by about 19%, which agrees closely with the 25% reduction reported previously (16) for the residual esterolytic activity of trypsin in an $\alpha_2$M-trypsin complex. Addition of SBTI (400 µg/ml) alone totally abolished the esterolytic activity of trypsin. When $\alpha_2$M was added before SBTI, the esterolytic activity was protected up to a molar ratio (trypsin to $\alpha_2$M) of about 1.15 (Fig. 8). When the maximum binding capacity of $\alpha_2$M was exceeded, a decrease in the SBTI-resistant trypsin activity occurred, apparently because of destruction of $\alpha_2$M by excess trypsin (21). The value of 1.15 agrees well with values of 0.92 and 1.25 reported previously as the molar ratio of trypsin to $\alpha_2$M that is close to the normal binding capacity of $\alpha_2$M (3, 21). In contrast, results for $\alpha_2M_f$ preparations indicated a major reduction in the functional capacity of the $\alpha_2$M in these preparations (Fig. 8). The esterolytic activity of trypsin was only protected up to a molar ratio of about 0.52, indicating that $\alpha_2M_f$ preparations contain $\alpha_2$M that has been previously proteolytically cleaved or is already complexed to a proteolytic plasma enzyme.

## ISOELECTRIC FOCUSING OF PURIFIED $\alpha_2M_f$ AND $\alpha_2$M

Further evidence that $\alpha_2M_f$ is a proteolytically derived fragment of $\alpha_2$M was obtained by electrofocusing. Previous studies by Jones *et al.* (23) showed that treatment of $\alpha_2$M with urea causes the macroglobulin to disassociate into both intact subunit monomers (mol wt 185,000) and subunit dimers (mol wt 340,000) (21). Since untreated $\alpha_2$M would not focus in a 5% polyacrylamide gel, apparently because of its large size (mol wt 670,000) (21), purified preparations of $\alpha_2$M were treated with 6 M urea before electrofocusing. $\alpha_2$M pretreated with urea showed four major bands, all with pI values lower than those of bands 1 and 2 from urea-treated or untreated $\alpha_2M_f$ preparations (Fig. 9). However, treatment of $\alpha_2$M preparations with trypsin and then urea produced a banding profile similar to that of urea-treated $\alpha_2M_f$ (Fig. 9).

## $\alpha_2$M AND ARGININE ESTERASE ACTIVITY

As shown previously (Fig. 3, Table 1), analysis of whole activated plasma samples from CF homozygotes and obligate heterozygote carriers by isoelectrofocusing indicated a quantitative deficiency in bands 1 and 2. The preceding results indicate that these bands represent fragments of $\alpha_2$M derived from proteolytic cleavage of the parent molecule. To determine whether a deficiency in $\alpha_2M_f$ production by the majority of CF homozygote and obligate heterozygote samples might be due to decreased levels of $\alpha_2$M, we quantitated $\alpha_2$M levels by single radial immunodiffusion. No significant difference between $\alpha_2$M levels in obligate heterozygote carriers and normal controls was found. CF homozygotes showed slightly increased $\alpha_2$M levels but all levels were within the normal range for $\alpha_2$M (Table 1).

$\alpha_2$M purified from two CF homozygote donors (C7422 and C2045 in Table 1) was nearly identical with $\alpha_2$M from normal controls when analyzed by SDS-polyacrylamide gel electrophoresis, both before and after reduction with DTT. Thus, CF $\alpha_2$M apparently is not abnormal in molecular weight or gross subunit structure. The same CF $\alpha_2$M preparations did, however, have slightly reduced binding capacity for trypsin relative to normal $\alpha_2$M (1.00 vs. 1.15 mol trypsin/mol $\alpha_2$M). This difference accounts for a difference of 7 µmol TAME hydrolyzed/min/mg $\alpha_2$M.

An alternative explanation for the observed deficiency in bands 1 and 2 was a deficiency in proteolytic cleavage of $\alpha_2$M, possibly because of a decreased level of active proteolytic (esterolytic)

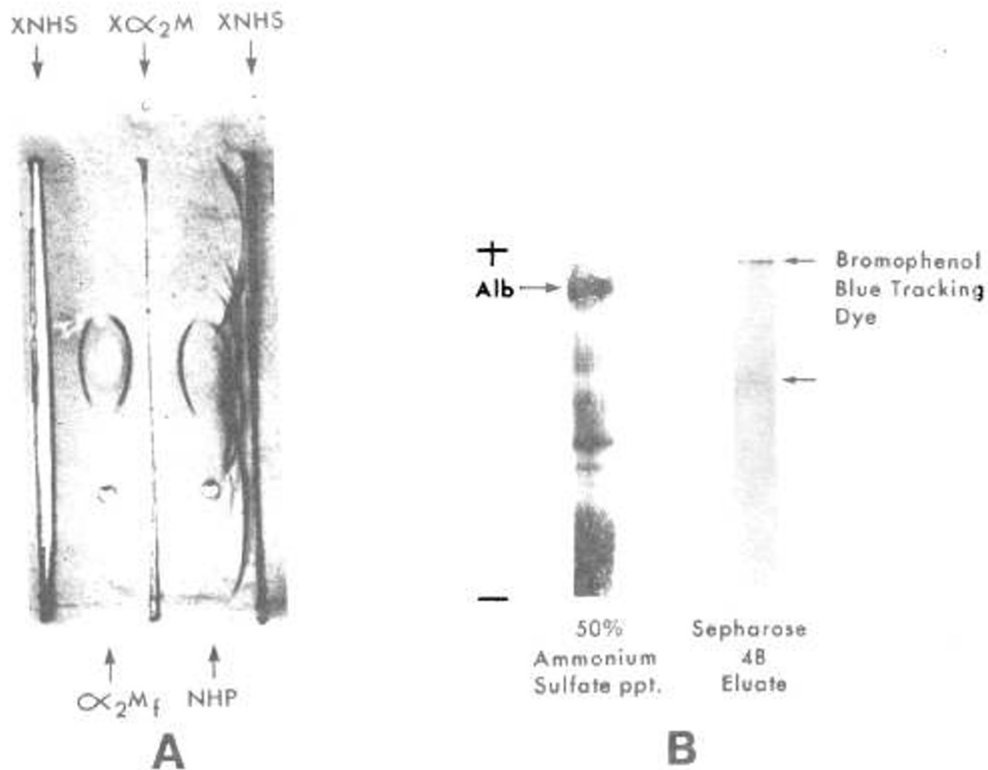


Fig. 6. *A*: results of analysis of Sepharose 4B eluate (6 mg/ml) by immunoelectrophoresis. Patterns were developed with rabbit-anti-whole human serum (*XNHS*) or rabbit-anti-$\alpha_2$-macroglobulin (*X*$\alpha_2$*M*) as indicated. The patterns for whole normal human plasma (*NHP*) are also shown. *B*: results of electrophoresis of 20 $\mu$l Sepharose 4B eluate (6 mg/ml) and 20 $\mu$l 50% precipitable proteins (50 mg/ml). The position of albumin is indicated.

enzyme activity in CF plasma. Therefore, we analyzed all samples from CF homozygotes, heterozygote carriers, and normal controls for total arginine esterase activity (esterolytic activity) in the presence and absence of SBTI (400 $\mu$g/ml). Samples from CF homozygotes and obligate heterozygote carriers showed decreased levels of total arginine esterase activity and decreased levels of SBTI-inhibited esterolytic activity as compared with the normal control samples (Table 1). Examination of samples indicated that, with the exception of CF samples found to have a strong band 5 (Fig. 3, Table 1), there appeared to be a correlation between decreased arginine esterase activity and a decrease observed in the $\alpha_2M_f$ bands. Examination of the SBTI-resistant and SBTI-inhibited activities of CF samples with a strong band 5, in each case revealed a larger amount of SBTI-resistant activity relative to SBTI-inhibited activity in these samples as compared with normal control samples. There was no obvious correlation between age, sex, or severity of the disease and abnormalities in IEF banding profiles of CF samples in these preliminary studies. However, further studies with samples from more patients are required before definite conclusions can be drawn.

## DISCUSSION

Harpel (21) has shown that the subunit structure of $\alpha_2M$ is altered by interaction with the proteolytic enzymes it inhibits. $\alpha_2M$ that has not formed a complex with a protease consists of four subunit chains of molecular weight approximately 185,000 each. Upon complex formation with trypsin, plasmin, thrombin, kallikrein, and possibly other plasma proteases, $\alpha_2M$ is proteolytically cleaved at the same region in each subunit chain, resulting in the production of a single proteolytic derivative with a molecular weight approximately half that of the subunit chain (85,000) (Fig. 7).

Our results suggest that $\alpha_2M_f$ may be this proteolytic subunit derivative of $\alpha_2M$. Evidence that $\alpha_2M_f$ does not represent a plasma protease bound to $\alpha_2M$ during our isolation procedure is derived from the results showing that plasma proteases do not have pI values near 5.5 (24); that plasminogen was not found associated with preparations of $\alpha_2M_f$ as assessed by CIE; and that $\alpha_2M_f$ could be derived from intact $\alpha_2M$ by treatment with trypsin or with thrombin (49). The fact that $\alpha_2M_f$ preparations will focus in a 5% polyacrylamide gel with 4 M urea added, whereas intact $\alpha_2M$ will not, may be related to the observation that intrachain disulfide bonds in $\alpha_2M$ subunits which have been cleaved by proteolysis may be more susceptible to denaturation by urea (23).

$\alpha_2M$ appears to participate in the regulation of several circulating enzyme systems, as it forms complexes with plasmin (18, 19, 21, 24), thrombin (26, 46), and kallikrein (20). In addition, $\alpha_2M$ has been found to bind trypsin, chymotrypsin, cathepsins, and other endopeptidases (3, 21). Figure 10 shows a generalized diagram of the possible points in the hemostatic, inflammatory, and complement pathways where $\alpha_2M$ has been shown to interact and possibly function as a regulatory agent (24, 40, 41). The double-headed arrows leading to and from $\alpha_2M$ indicate the points of interaction of $\alpha_2M$ with these systems.

Before activation of Hageman factor by glass, ellagic acid, or kaolin (40), the plasma proteases (esterases) are in an inactive or proenzyme form (21, 24, 40) and will not interact with $\alpha_2M$ (3, 16, 21). Therefore, $\alpha_2M$ is not proteolytically cleaved, and proteolytic derivatives ($\alpha_2M_f$) cannot be observed by isoelectric focusing of whole plasma (Fig. 2). When Hageman factor is activated it can initiate the conversion of the proteases to active forms capable of complexing to $\alpha_2M$ and of interacting with systems that generate kinin and anaphylatoxin (Fig. 10) (23, 24, 40, 41).

In activated plasma samples from most CF homozygotes and heterozygote carriers, decreased amounts of $\alpha_2M_f$ are noted by isoelectric focusing (Table 1). The finding that 3 of 12 CF patients were not markedly deficient in $\alpha_2M_f$ (Fig. 3, Table 1) may be further evidence of genetic heterogeneity in the clinical entity known as CF. The occurrence of a strong band 5, an extra band

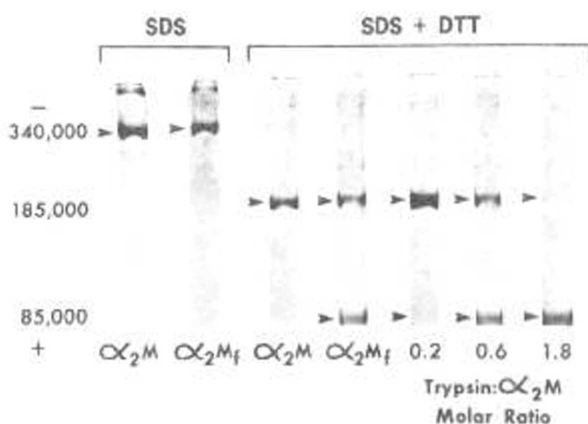


Fig. 7. Results of SDS-polyacrylamide gel electrophoresis of $\alpha_2$-macroglobulin ($\alpha_2M$) and $\alpha_2$M fragment ($\alpha_2M_f$). $\alpha_2$M (2 mg/ml) or $\alpha_2M_f$ (2 mg/ml) preparations were mixed 1:1 with either 10 M urea plus 1% sodium dodecyl sulfate (*SDS*) or reduced with a similar solution containing 14 mM dithiothreitol (*DTT*). Although not shown in this figure, at times unreduced $\alpha_2M_f$ preparations showed small amounts of the 185,000 molecular weight band. The results of prior treatment of $\alpha_2$M (2 mg/ml) with trypsin at different trypsin to $\alpha_2$M molar ratios are also shown. The highest concentration of trypsin was 88 $\mu$g corresponding to a trypsin to $\alpha_2$M molar ratio of 1.8. The approximate molecular weight of bands obtained before and after reduction is indicated. Twenty-microliter samples were analyzed.

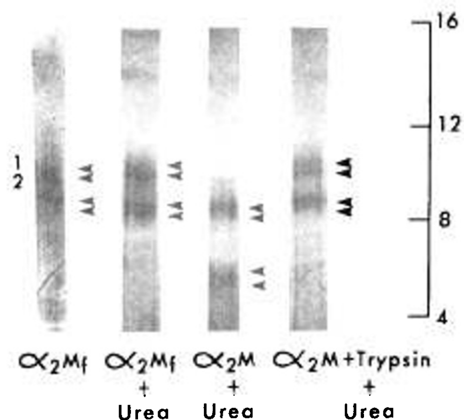


Fig. 9. Results of isoelectric focusing of 100 $\mu$l $\alpha_2$-macroglobulin fragment ($\alpha_2M_f$); 100 $\mu$l $\alpha_2M_f$ plus urea; $\alpha_2$-macroglobulin ($\alpha_2M$) plus urea; and 100 $\mu$l $\alpha_2$M plus trypsin (trypsin to $\alpha_2$M molar ratio of 1.5) plus urea. The final concentration of $\alpha_2$M was 2 mg/ml in each case. The final urea concentration used was 6 M. The centimeter scale shows the distance from the anode.

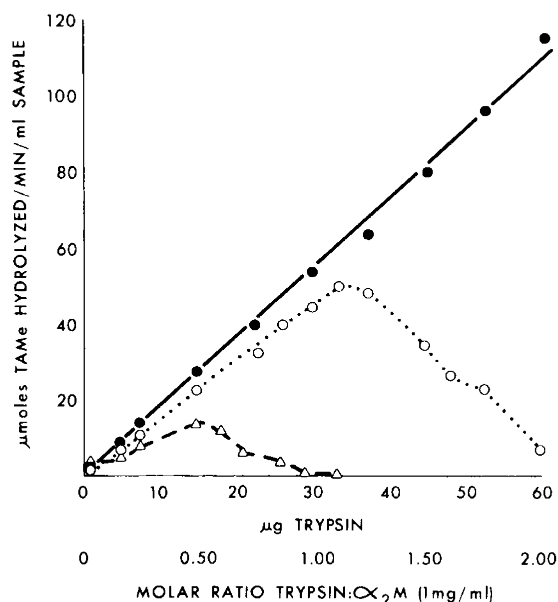


Fig. 8. Hydrolysis of tosyl arginine methyl ester (*TAMe*) by trypsin. ●——●: amount TAME hydrolyzed by trypsin alone. O——O: TAME hydrolyzed by mixtures of trypsin plus $\alpha_2$-macroglobulin ($\alpha_2M$)(1 mg/ml) plus soybean trypsin inhibitor (SBTI) (400 $\mu$g/ml). Δ- -Δ: TAME hydrolyzed by mixtures of $\alpha_2$M fragment preparations (1 mg/ml) plus trypsin plus SBTI (400 mg/ml).

above band 5, and an abnormally high SBTI-resistant fraction suggests that these patients are not normal *per se*. Since $\alpha_2$M levels are normal in CF homozygotes and obligate heterozygote carriers, this finding suggests a decreased proteolysis of $\alpha_2$M in plasma from such individuals. Decreased proteolysis of $\alpha_2$M may be due to several different mechanisms, which are not necessarily mutually exclusive. Among these possibilities are the following: (*a*) Plasma proteases in general may not be activated to levels found in normal plasma, because of an abnormality in Hageman factor or in the activation of Hageman factor. (*b*) A specific protease may be abnormal structurally or may remain partially in proenzyme form. (*c*) There may be a genetically determined abnormality in the structure of $\alpha_2$M, primarily at the active site, thus decreasing its binding affinity for the plasma proteases. (*d*) The active site of $\alpha_2$M may be blocked by an inhibitor, reducing its interaction with the proteases. (*e*) The active site of the proteases may be blocked either before or after their conversion to active forms, inhibiting their ability to interact with $\alpha_2$M.

Evidence for possibilities *a*, *b*, and *e* is found in the work of Rao *et al.* (39) and Rao and Nadler (38) and in the results of this study (Table 1), which collectively point to a deficiency in arginine esterase activity in CF plasma. This esterase activity is thought to be due primarily to plasma kallikrein activated by Hageman factor (9), although no attempt has been made to exclude a contribution to this activity by plasmin or other plasma proteases which are known to have esterolytic activity and can hydrolyze TAME (40).

Possibilities *c* and *d*, however, seem to us to be more attractive. A structural abnormality in, or a blockage of, the binding of $\alpha_2$M could decrease the inhibition of kallikrein, plasmin, and plasminogen activator (among other plasma proteases). $\alpha_2$M is thought to be the major inhibitor of plasmin (24). Plasmin, if not effectively inhibited, could participate in increased activation of kallikrein and thus increased production of kinins brought about by the conversion of kininogen to kinin. In addition, increased production of C3a and chemotactic factors could occur through the action of plasmin on C3 and C1s (Fig. 10) (24, 40, 41).

Conover *et al.* (13) have suggested that the CF factor may be a C3a or kinin-like molecule and have speculated how this molecule could cause the pathophysiology of CF. Considerable evidence now suggests that the CF factor binds or complexes to IgG (2, 4, 50). Evidence that C3a can complex to IgG has also been reported (35). In the hypothesis of Conover *et al.* (13) and in a similar theory which we put forth (48), once CF factor or C3a is complexed to IgG it is protected from its inhibitor. Conover *et al.* assumed that a defect in the anaphylatoxin inactivator (AI) or another enzyme similar to carboxypeptidase B must occur in C/F to explain the accumulation of C3a or CF factor. However, a defect in AI need not be postulated, if C3a is protected from AI when it is complexed to IgG. Furthermore, Lieberman (29) has shown recently that carboxypeptidase B-like activity is normal in CF serum.

Instead of a defect in AI or a similar exopeptidase, we suggest that the CF factor, if it is C3a or kinin-like material, may be overproduced in CF due to the combined production of C3a and kinin by the classic inflammatory and complement pathways and

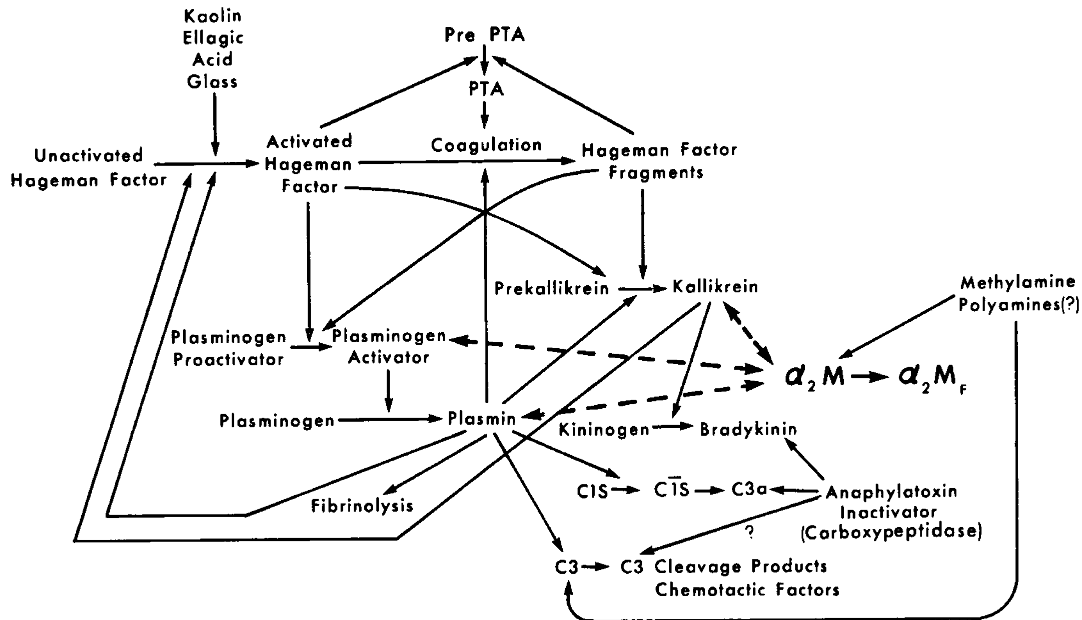


Fig. 10. Diagram illustrating the points of interaction of $\alpha_2$-macroglobulin ($\alpha_2M$) with the hemostatic, inflammatory and complement pathways and the interrelationship of these systems (modified from Kaplan (24) by permission of *Microvascular Research*). $\alpha_2M_f$: $\alpha_2$M fragment; *PTA:* plasma thromboplastin antecedent.

as a result of abnormally elevated plasmin-like activity brought about by an abnormality in $\alpha_2$M, its primary plasma inhibitor. If a defect in $\alpha_2$M is responsible indirectly for the pathophysiology of CF, then the etiology of the disease would be roughly analogous to that of heredity angioneurotic edema, in that a deficiency in C1 esterase inhibitor has been found to be indirectly responsible for the pathophysiology of that disease (41). A concomitant decrease in total arginine esterase activity or kallikrein activity in CF might be explained by assuming (1) hyperconversion of prekallikrein to kallikrein, brought about by plasmin (40), with subsequent inhibition of kallikrein by $\alpha_1$ antitrypsin or C1 esterase inhibitor (24, 40); (2) feedback inhibition of Hageman factor activation by uninhibited plasmin; or (*3*) a concurrent defect in kallikrein, or another protease, but not plasmin.

Although a structurally abnormal active site on $\alpha_2$M could explain a decrease in its proteolysis, blockage of the active site would have a similar effect. Inhibition of trypsin and other plasma proteases by SBTI and presumably by $\alpha_2$M may involve proteolytic cleavage at a single arginine or lysine residue in the inhibitor, located within a peptide loop formed by intrachain disulfide bridging (21, 27). A covalent bond is then formed between the enzyme and the inhibitor, involving an acyl enzyme or tetrahedral intermediate, in which a serine residue at the active site of the protease (trypsin) forms an ester bond with the carboxyl of an arginine or lysine residue at the active site of the inhibitor (21, 27). It is known that methylamine can destroy the ability of $\alpha_2$M to complex with plasma proteases (44, 47). In fact, methylamine at concentrations of about 0.25 M causes the breakdown of $\alpha_2$M into smaller protein derivatives with faster electrophoretic mobility (3, 44, 47). In preliminary studies (49), we have found that methylamine causes no gross structural changes in $\alpha_2$M at concentrations of 0.05 M or lower (as assessed by isoelectric focusing and SDS-polyacrylamide gel electrophoresis), whereas methylamine causes a reduction in the trypsin binding capacity of $\alpha_2$M. The binding site of methylamine to $\alpha_2$M has not been identified, but binding may occur near or at the lysine or arginine that is the site of cleavage by the serine proteases. Further research will be required to fully answer this problem.

It has been recently shown that CF may involve an abnormality in polyamine metabolism (8, 36, 43) and that excess spermidine is found in the plasma of CF patients (36). It might be that this polyamine or a derivative of it could bind to the active site of $\alpha_2$M in a manner analogous to that of methylamine and thus cause a decrease in the effective binding affinity of $\alpha_2$M for proteases. Alternatively, it might bind to one or more proteases, altering their activity and binding affinity for $\alpha_2$M. Experiments concerned with assessing the possible inhibitory effect of polyamines or their derivatives on the functional activity of $\alpha_2$M and selected plasma proteases are currently being pursued in this laboratory and will be published in a subsequent report (49).

## CONCLUSION

The results of this investigation have shown that activated plasma samples from CF patients and obligate heterozygote carriers for CF are deficient in a derivative of $\alpha_2$M, which results presumably from proteolysis of intact $\alpha_2$M subunits by plasma proteases. Several mechanisms are hypothesized to explain this deficiency. The most attractive explanations are either (*1*) a structurally abnormal active site on CF $\alpha_2$M, which would result in a decreased binding affinity of CF $\alpha_2$M for the plasma protease, or (*2*) blockage of the active site of CF $\alpha_2$M by polyamine derivatives which are known to be found in elevated amounts in CF plasma.

Certainly further research is indicated to explore the possible relationship of an abnormality in polyamine metabolism to a deficiency in proteolysis of $\alpha_2$M and to elucidate the involvement of either defect to the pathophysiology of cystic fibrosis. However, it may well be that an abnormality in $\alpha_2$M-protease interactions, possibly coupled with an abnormality in polyamine metabolism, could explain the presence of CF factor(s) in cystic fibrosis.

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