

## A MOLECULAR ABNORMALITY OF URINARY MUCOPROTEIN IN CYSTIC FIBROSIS OF THE PANCREAS \*

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Since its first complete description in 1938 by Anderson, concepts of the pathogenesis of cystic fibrosis have been rapidly and continuously modified. The initial concept of pathology primarily in the pancreas had quickly to be extended to include primary pathology in other mucous-secreting glands because of clinical and histological evidence of abnormalities in the biliary tract and especially in the lungs. With the demonstration of the elevation of sweat electrolytes in this disorder, even this concept proved to be too limiting and had to be enlarged to include exocrine glands in general.

It is the purpose of this report to demonstrate that the molecules of the urinary mucoprotein of Tamm and Horsfall have an abnormal structure in this disease. In this way we wish to present evidence pointing to the involvement of an organ not hitherto implicated in cystic fibrosis and, more importantly, evidence indicating the molecular nature of the fundamental abnormality in this hereditary disease.

### METHODS

Urinary mucoprotein was prepared in several ways. T & HE, the total urinary mucoprotein of Tamm and Horsfall, was prepared as described elsewhere (1) by twice precipitating with 0.58 M NaCl, dispersing in water, and dialyzing against water. As has been pointed out previously (2), urinary mucoprotein forms tactoids in the presence of dilute salt. Similar aggregates can be centrifuged from untreated urine (International refrigerated centrifuge) at 4,000 rpm for 20 minutes. This urinary sediment was resuspended in water and, after dialysis against water, again centrifuged to remove the water-insoluble material. The clear supernate was brought to 0.58 M with sodium chloride. The precipitate which then formed was removed by centrifugation, re-

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suspended in water, and dialyzed against water. This preparation thus contained only that portion of T & HE that was aggregated in urine, and is designated T & HE (sedimented).

To the supernatant urine remaining after the aggregates had been centrifuged off, NaCl was added to increase the molarity by 0.58. A small sediment formed which was taken up in water and dialyzed against water as before. This preparation is designated T & HE (dispersed).

Viscosity studies were made in a modified Ostwald capillary viscometer. All measurements were made in a temperature-controlled water bath at 20° C.

Ultracentrifugal studies were made in a Spinco model E ultracentrifuge in which the temperature was also controlled at 20° C.

Mucoprotein concentrations were determined from refractive index measurements made in a Brice-Phoenix differential refractometer which had been calibrated against dry weights of the normal mucoproteins.

Ultraviolet spectra were determined in a Beckman recording spectrophotometer.

### RESULTS

The urinary mucoprotein of Tamm and Horsfall can be isolated in relatively pure form by very mild procedures from normal human urine. This mucoprotein, which temporarily inhibits the hemagglutination reaction of the myxoviruses, has been shown (3) to exist in two molecular forms under physiological conditions. The smaller, T & HE7, of these flexible rod-shaped molecules has a molecular weight of  $7 \times 10^6$ , a length of  $6 \times 10^3 \text{Å}$ , and a width of  $40 \text{Å}$ . The larger T & HE28 is comprised of four of the smaller ones, two side by side and two end to end, to make a molecular weight of  $28 \times 10^6$ , a length of  $12 \times 10^3 \text{Å}$ , and a width of  $80 \text{Å}$ .

It has been possible (2) by means of ethanol to split the molecules of T & HE7 transversely into halves. These halves have been isolated in relatively pure form and characterized as rod-shaped molecules (T & H DF) of molecular weight  $3.5 \times 10^6$ , length  $3 \times 10^3 \text{Å}$ , and width  $40 \text{Å}$ . Still smaller fragments (T & HE $\rightarrow$ DF) of molecular weight



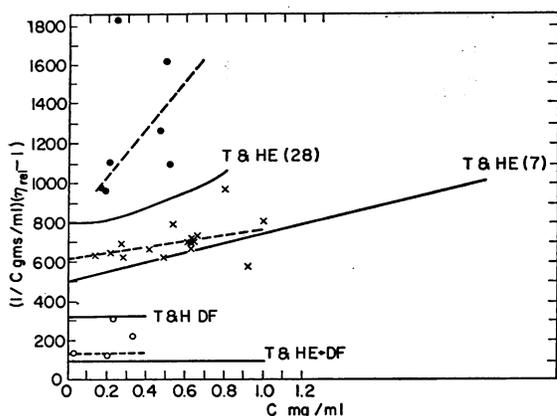


FIG. 1. VISCOSITY STUDIES ON THE MOLECULAR FORMS OF URINARY MUCOPROTEIN IN CYSTIC FIBROSIS COMPARED WITH NORMAL CONTROLS. The solid curves from top to bottom represent T & HE28, T & HE7, T & H DF, and T & HE $\rightarrow$ DF from normal controls. See References 1-3 for the experimental points from which these curves were determined. The solid dots are measurements from T & HE(sedimented)CF immediately after preparation. The crosses are from T & HE(sedimented)CF after several days' wait or after reprecipitation with NaCl. The open circles are from T & HE(dispersed)CF. The dashed lines are fitted to each group of experimental points.

Although each of these points has been determined on a sample from a separate individual rather than on a dilution series of a single sample, the points show a steeply rising viscosity with increasing concentration which extrapolates on dilution toward the approximate intrinsic viscosity intercept of normal specimens. This suggests that at great dilution the molecular form T & HE28 CF corresponding to T & HE28 exists, although the data are not sufficient to permit the estimation of an intrinsic viscosity or an axial ratio. However, at higher concentrations the degree of molecular aggregation is much greater than normal. The elevated viscosity in this type of sample appears frequently, but not invariably, in our series of 12 patients. The precise conditions necessary to obtain the very high viscosities have not yet been determined.

The viscosity of T & HE(sedimented)CF drops rapidly to about 620. This fall in viscosity is much more rapid than in normal controls which take weeks to months at a concentration of 1 mg per ml, depending on the salt concentration, to drop to an intrinsic viscosity of 500. This indi-

cates that the polymerized form of T & HE28 CF and the T & HE28 CF molecule itself are relatively unstable. Plotted as crosses in Figure 1 are viscosities measured on samples of T & HE(sedimented)CF from cystic fibrosis patients after standing for several days or after reprecipitating with 0.58 M NaCl. These include measurements made on the same samples that gave the very high viscosities also shown in Figure 1 when measured immediately after preparation. The very rapid drop in viscosity following a second sodium chloride precipitation suggests an unusual sensitivity to salt. The viscosity of these preparations has not been observed to go below the viscosity of T & HE7 CF. It can therefore be concluded that T & HE7 CF in cystic fibrosis is a relatively stable molecule at the concentrations for which data are available.

The viscosity data for T & HE7 CF extrapolate to an intrinsic viscosity of 620, somewhat higher than the normal value of 500. Therefore, the axial ratio of T & HE7 CF is greater than normal and the molecules are longer than normal.

Evidence has been reported (4) that the formation of coacervates may be useful in the purification of larger proteins. In preparations of normal urinary mucoprotein the viscosity of T & HE(sedimented), corresponding to the coacervate phase, in fresh preparations approximates that of T & HE28. The viscosity of T & HE(dispersed) is generally lower, approximating that of T & HE7. In older preparations of both types, the viscosities drop toward that of T & HE7. The distribution of T & HE between the sedimented and dispersed phases varies with the specific gravity of the urine (5) and with the time and temperature at which the urine is stored before processing. In normal pooled preparation from adults made in the manner of the cystic fibrosis preparations, 1 to 3 mg per L of the total T & HE has been found in the dispersed phase. In samples from two patients with cystic fibrosis the yield of urinary mucoprotein in the dispersed phase was 3.98 and 2.99 mg per L. Of four patients with cystic fibrosis the yield of T & HE(sedimented)CF had a mean of 11.6 mg per L (range, 11 to 12.6).

The viscosity of T & HE(dispersed)CF, plotted as open circles in Figure 1, is markedly lower than that of T & HE7. It appears, therefore, that the

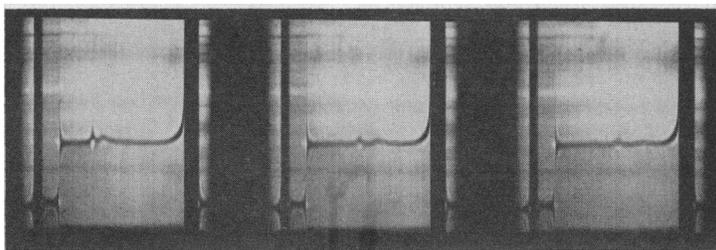


FIG. 2. ULTRACENTRIFUGAL RUN ON T & HE CF IN H<sub>2</sub>O IMMEDIATELY AFTER PREPARATION. Photos at 4- and 2-minute intervals, 59,780 rpm, bar angle 45°, S = 33.1 for T & HE7 and 49.8 for T & HE28 CF.

formation of coacervates in urine does indeed involve a selection or purification of T & HE molecules and that shorter, incomplete fragments of urinary mucoprotein molecules are left behind in the dispersed phase. The viscosities of samples of T & HE (dispersed) CF show considerable scatter in Figure 1 and approximate but are somewhat higher than the value corresponding to T & HE → DF. The intrinsic viscosity of T & HE (dispersed) CF is 125.

Confirmatory evidence for the above conclusions was sought through a series of ultracentrifugal studies. Figure 2 shows an ultracentrifugal pattern of freshly prepared T & HE from a cystic fibrosis patient. The pattern is typical of that of normal T & HE in that two peaks appear. The slow sharp peak corresponds in shape and position to that of normal T & HE7. The faster peak corresponds to T & HE28 in its position, shape, and the presence of the boat-shaped baseline below the peak. Figure 3 shows an ultracentrifugal pattern of T & HE CF several days after preparation; it shows that the urinary mucoprotein has converted entirely to T & HE7 CF. A dilution series in water was prepared from this material, the ultracentrifuge data from which are shown in Figure 4

along with data from several other samples of T & HE7 CF.

The data from T & HE7 CF are plotted as crosses in Figure 4 for comparison with sedimentation velocities of normal preparations of T & HE7 plotted as dots. All runs were made in water because of the poor solubility of T & HE in salt solution. It is seen that at each concentration the sedimentation velocity of the cystic fibrosis mucoprotein is faster than normal. The extrapolated sedimentation constant and the most dilute measured sedimentation velocity of T & HE7 CF is about 35 svedbergs (S), higher than the normal value of 29.5S (3). This sedimentation constant combined with the corresponding intrinsic viscosity of 620 from Figure 1 yields a molecular weight of  $1.0 \times 10^7$  for the molecules of T & HE7 CF, calculated with the same assumptions used for T & HE7. The notation T & HE7 CF will, however, be retained to identify these molecules with those of T & HE7 which have a molecular weight of  $7 \times 10^6$ . T & HE7 CF appears to be a stable molecule which does not dissociate further even on standing for about 1 year in urine.

To examine the behavior of T & HE CF, a single sample of this urinary mucoprotein was

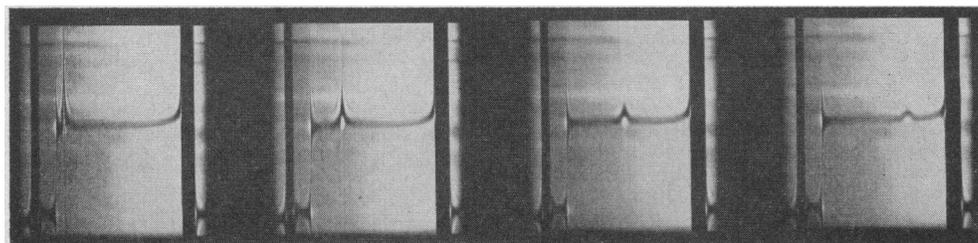


FIG. 3. ULTRACENTRIFUGAL RUN ON T & HE (SEDIMENTED) CF IN H<sub>2</sub>O 27 DAYS AFTER PREPARATION. Photos every 8 minutes, 59,780 rpm, bar angle 35°, S = 21.0.

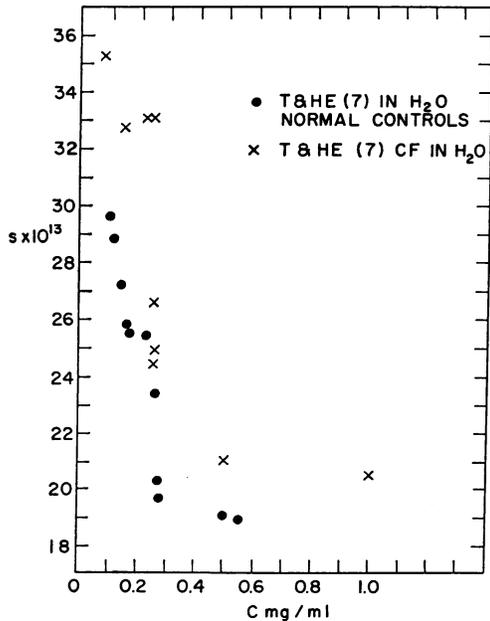


FIG. 4. ULTRACENTRIFUGAL STUDIES ON CYSTIC FIBROSIS URINARY MUCOPROTEIN T & HE7 CF FOR COMPARISON WITH NORMAL CONTROLS. All sedimentation velocities were determined at 20° C in water solution.

converted into T & HE→DF by the procedure described in Reference 1. An ultracentrifugal pattern of this preparation showed a single peak which in shape and position corresponded to normal T & HE→DF. This demonstrates that the bonds linking the T & HE→DF CF molecules together to make T & HE CF do not differ from normal in their ability to be split by alcohol. It has not yet been possible to obtain sufficient material to perform the indicated electrophoretic examination on this type of preparation.

The nature of the "fragments" of urinary mucoprotein molecules which appear in T & HE(dispersed)CF is uncertain. Viscosities determined on this preparation show considerable scatter, probably due to contamination with T & HE(sedimented)CF. However, most of the experimental points occur just above the value for normal T & HE→DF (Figure 1). It has proven extremely difficult to obtain sufficient material for ultracentrifugal studies; however, small peaks have been found on one plate that have a sedimentation velocity of 19.3S. Also, a peak with this sedimentation velocity appears in a preparation of T & HE CF which shows that this peak

moves considerably more slowly than the peak of T & HE7. The 19.3S peak is extremely small and sharp. The existence of a discrete peak indicates that the fragments are very much alike in size and shape and may be considered a distinct component. Examination of the sedimentation velocity data in References 1 and 2 for T & H DF and T & HE→DF in H<sub>2</sub>O shows that a sedimentation velocity of 19.3S is slightly faster than that to be expected of T & HE→DF. Combination of the viscosity and ultracentrifugal data yields a molecular weight of  $2.3 \times 10^6$  in contrast to  $1.7 \times 10^6$  for normal T & HE→DF. It is probably more than coincidence that this is one-quarter of the molecular weight of T & HE7 CF.

Electron microscopic studies of urinary mucoprotein in cystic fibrosis are demonstrated in Figure 5; 5A is an electron micrograph of a sample of normal T & HE showing many of the long thick fibers typical of T & HE28. This should be compared with 5B which shows at the same magnification the aggregated clumps of T & HE(sedimented)CF. The clumps shown are rather more prominent than is usually seen in similar micrographs, and they may account for the very high viscosity noted above. Figure 5C shows that T & HE(dispersed)CF is composed of many short fragments which have not been observed to this extent in normal preparations. The thickness of the fibers that can be resolved in both sedimented and dispersed preparations of cystic fibrosis mucoprotein is much more commonly the 40Å of T & HE7 than the 80Å of T & HE28. Measurements on the electron micrographs of T & HE(dispersed)CF show the predominance of particles somewhat longer than the 1,500Å of normal T & HE→DF, in agreement with the ultracentrifugal and viscosity measurements.

The ultraviolet spectrum has been examined on samples of urinary mucoprotein from four patients with cystic fibrosis. The main peak and its fine structure correspond completely to that of normal urinary mucoprotein, and the  $E^{1\%}_{1cm}$  is normal. Additional peaks appear occasionally in the spectra from cystic fibrosis patients, but these have been correlated with the antibiotic therapy which the patients were receiving. Apparently some excretion products of antibiotic metabolism are bound to urinary mucoprotein.

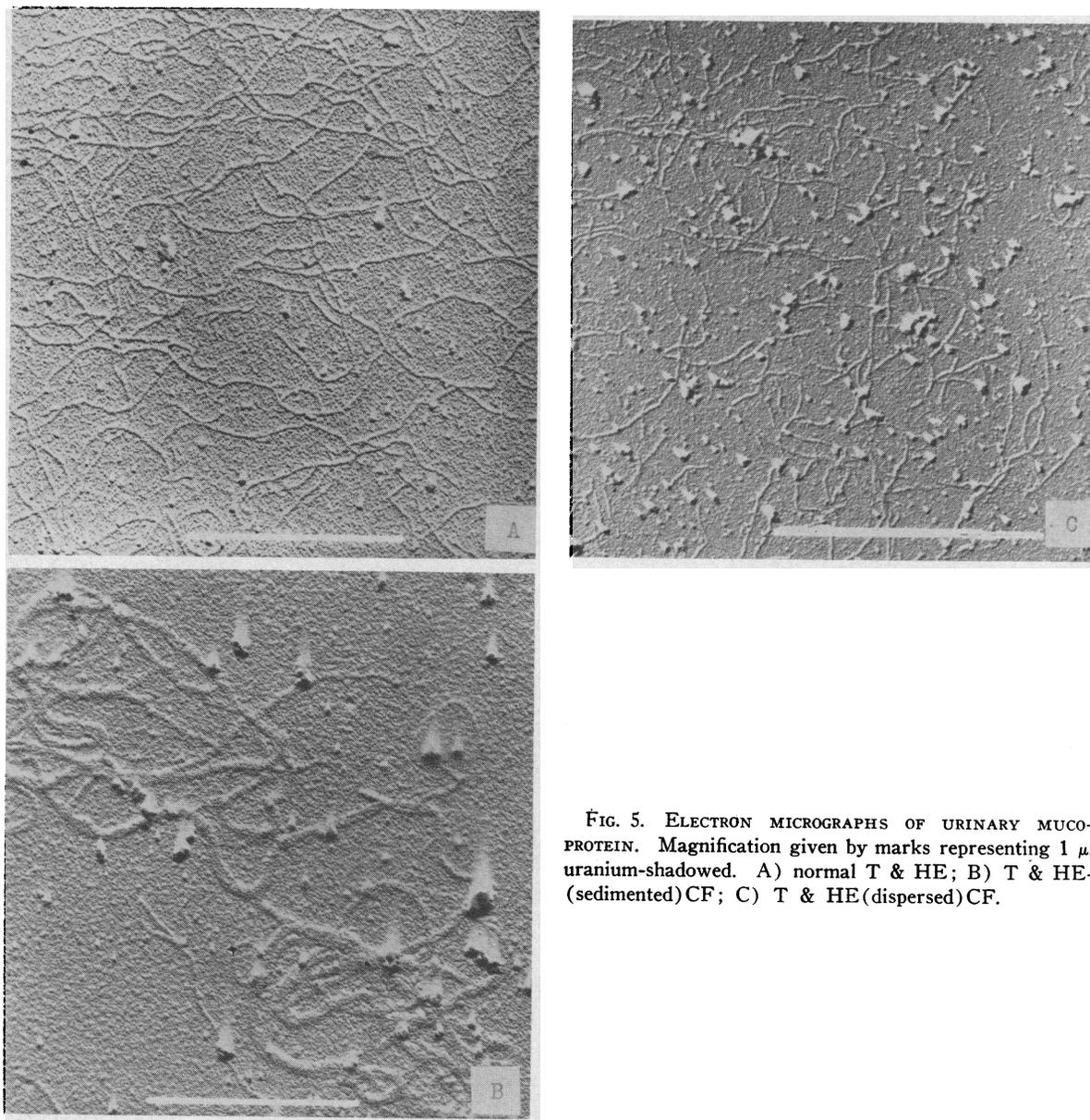


FIG. 5. ELECTRON MICROGRAPHS OF URINARY MUCOPROTEIN. Magnification given by marks representing  $1 \mu$ , uranium-shadowed. A) normal T & HE; B) T & HE-(sedimented)CF; C) T & HE(dispersed)CF.

The pH of T & HE CF in water solution varies about 6.0, which is within the normal range.

#### DISCUSSION

The evidence from viscosity, ultracentrifugal, and electron microscopic studies is consistent in pointing to the existence in cystic fibrosis of an abnormally highly aggregated form of urinary mucoprotein. This is a very unstable aggregate of T & HE28 CF which has an intrinsic viscosity of over 800. The molecule of T & HE28 CF is

itself unstable and dissociates on standing and more rapidly in the presence of NaCl into molecules of T & HE7 CF.

The molecules of T & HE7 CF are abnormally long, having an intrinsic viscosity of 620, a sedimentation constant of 35S, and a molecular weight of  $1.0 \times 10^7$ . These molecules are stable on standing and in the presence of salt.

In addition to the above abnormal molecular forms of urinary mucoprotein, there exists in the dispersed phase of the urine in cystic fibrosis a

small amount of smaller molecular "fragments" of uniform size, with an intrinsic viscosity of 125, and a measured sedimentation velocity of 19.3S. The nature of these fragments is uncertain, but the measured properties are closer to those of T & HE  $\rightarrow$ DF than to those of T & H DF. The fragments have been identified with urinary mucoprotein only by their solubility in salt solution and by their similarity in size and shape to T & HE  $\rightarrow$ DF. It is, of course, possible that this component represents an entirely new macromolecule peculiar to cystic fibrosis. The simpler hypothesis is to link the fragments with the abnormal urinary mucoprotein.

These experimental results may be given a uniform interpretation by assuming the existence of a metabolic error in the synthesis of urinary mucoprotein; this results in an excess of fragments that cannot be completely incorporated into the final molecules, which in turn results in the synthesis of abnormal urinary mucoproteins that tend to polymerize indiscriminately but with less than the usual stability.

Highly viscous mucoid substances have been previously reported in cystic fibrosis (6). The reduction of the high viscosity by sodium chloride has also been reported (7). The significance of the present report is the demonstration of the increased viscosity due to aggregation and an abnormality in structure of a purified, easily available, and well characterized mucoprotein illustrating the molecular nature of the fundamental abnormality in this disease.

The most striking deviations from the normal occur in measurements performed upon T & HE (dispersed)CF. The small quantity of this material usually available in urine samples frequently makes it impossible to perform the technically simple viscosity measurement. Electron microscopy can, however, be done on very small samples, and here also the deviations from the normal are marked. It would be interesting if, on the repeated examination of greater numbers of patients, the electron microscope maintained its reliability as a diagnostic tool in cystic fibrosis.

The relationship of a molecular abnormality in this virus-inhibiting mucoprotein to the increased susceptibility to infection in patients with cystic fibrosis is not clear at this time.

The normal control data presented have been obtained from adults. All of the normal viscosity data have been checked with normal children similar in age to the cystic fibrosis patients. The normal viscosity data require no revision.

The demonstrated tendency of the cystic fibrosis mucoprotein to form aggregates might influence the physicochemical data in the direction of higher molecular weights. This influence should be minimized by working with very dilute water solutions without salt, as was done here.

#### SUMMARY

The several molecular forms of urinary mucoprotein of Tamm and Horsfall obtained from cystic fibrosis patients have been examined by viscosity, ultracentrifugal, and electron microscopic observations. The two normally occurring forms of this fibrous mucoprotein, T & HE28 and T & HE7 (of molecular weights 28 and 7 million, respectively), have been identified as T & H28 CF and T & H7 CF in cystic fibrosis urine. The larger form, T & HE28 CF, aggregates more readily but is much less stable than normal. It dissociates on standing and in the presence of salt into T & HE7 CF. The smaller form has an intrinsic viscosity of 620, a sedimentation constant  $s_{20,w}^0$  of 35, an average molecular weight of about 10 million, and is stable on standing.

In addition, a component of smaller "fragments" which has no naturally occurring normal counterpart is present in the dispersed phase of cystic fibrosis urine. This component has an intrinsic viscosity of about 125 and a measured sedimentation velocity of 19.3 svedberg units. It resembles in its physical properties T & HE  $\rightarrow$ DF, a component which has been prepared from normal urinary mucoprotein and which comprises quarter molecules of T & HE7. The molecular weight of the fragments is  $2.3 \times 10^6$ . These molecular abnormalities are characteristic of each of the cystic fibrosis patients examined and have not been found in hypertensive patients or in normal people who have been examined.

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