AN ELECTRON MICROSCOPE EXAMINATION OF URINARY MUCOPROTEIN AND ITS INTERACTION WITH INFLUENZA VIRUS

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ABSTRACT

A hemagglutination-inhibitory mucoprotein from human urine has been studied with the electron microscope. It consists of filaments, with diameters of 40 to >240 A, composed of smaller fibrils. In the two-dimensional projection of the electron micrographs, the single fibrils often show a zig-zag course with a periodicity of 100 to 140 A; the single branch of a zig-zag measures about 60 A in length and either 20 or 40 A in width. Still thinner fibrillar elements are observable with diameters of 10 A or less. In three-dimensional aspect, the zig-zag structure might be a helix. The fibril-bundle (or filament) reveals a complicated configuration. Heat treatment at 70°C shows some indication of denaturation (*e.g.* filaments are shorter), whereas at 80°C almost complete degradation of the protein into individual zig-zag elements or smaller pieces is attained. The interaction between influenza virus particles and inhibitory mucoprotein consists of the attachment of a fiber molecule to the virus projections at several sites and frequently on more than one virus particle.

INTRODUCTION

In 1942 Hirst (1) described the phenomenon of the agglutination of red blood cells by influenza viruses. This reaction is inhibited by various mucoproteins such as serum mucoids (2–4), ovarian cyst mucin (5), ovomucin (6, 7), human red cell group substances (8, 9), ovine and bovine salivary mucin (10), and urinary mucoprotein (11).

The present paper is concerned with the morphology of the urinary mucoprotein, both normal and heat-inactivated, and the mechanism of hemagglutination (HA) inhibition as it can be seen in the electron microscope. The shape and structure of the molecules described here are rather different from those calculated and observed by other techniques.

MATERIALS AND METHODS

Urinary mucoprotein was prepared from human urine by precipitation in 0.58 M NaCl solution (12). The precipitate was washed 3 times in 0.58 M sodium chloride solution, centrifuged at 1900 rpm for 30 minutes, the sediment washed again 3 times in 0.58 M NaCl solution, and finally dialyzed against 800 times the volume of distilled water for 5 days. The dialyzed solution was centrifuged at 7000 RPM for 30 minutes, and the sediment discarded. The supernatant was frozen and dried.

The inhibitory mucoprotein (12) was extracted from the dried mucoprotein in 0.025 M phosphate buffer at pH 6.8 for 18 hours at 4°C. Its insoluble portion was spun down at 3800 *g* for 30 minutes. The supernatant was frozen-dried and kept at 4°C. Small portions of this were dissolved in distilled water (at least 10 hours at 4°C) before being used in a test.

The heat-treatment of the inhibitor solutions was carried out in a water bath at 70°C or at 80°C for 45 minutes.

The influenza strains, A/Singapore and A/WS grown on embryonated eggs, were used in allantoic fluid, with the titers of ELD_{50}/HA units $>10^6$ and stored at 1 to 2°C. In some experiments the infected allantoic fluid was centrifuged at 3000 *g* for 15 minutes, and the sediment discarded.

Red blood cells (rbc) from individual chickens were collected, treated with sodium citrate, washed

3 times in 0.85 per cent NaCl solution buffered with 0.01 M phosphate at pH 7.2, and used as 1 or 2 per cent suspensions in Ringer's solution.

For the hemagglutination reaction, two-fold dilution series were made of infected allantoic fluid in 0.2 ml Ringer's solution (pH 7.2), and 0.2 ml of the rbc suspension was added; readings were made after 60 minutes at 28°C.

The hemagglutination-inhibition reaction was carried out in two-fold or ten-fold dilution series of the mucoprotein in Ringer's solution. Following Tamm and Horsfall (12) the highest concentration used in the test was less than 100 μg of mucoprotein per milliliter to avoid the influence of viscosity. The final virus concentration was 4 and 8 HA units. Virus was heated at 56°C for 30 minutes to prevent elution in the test. The virus-inhibitor mixture was kept at 28°C for 1 hour before adding the rbc suspension (0.2 ml to each tube). Readings were made after 1 and 2 hours at 28°C. The end-points were determined by the highest dilution which showed complete inhibition.

For electron microscopy, unfixed material of inhibitor mucoprotein with a concentration approximately 90 μg/ml or 9 μg/ml, respectively, was negatively stained (13–15) in silicotungstate at pH 7.0 (35). An equal amount (9 μg/ml) of the virus suspension was added to the inhibitor solution and after 5 minutes negatively stained on the copper specimen grids which had been covered with carbon-coated holey Formvar films (16). The use of phosphotungstate and uranyl acetate for negative stain revealed similar results, but with lower contrast. The micrographs were made at primary magnifications of 40,000 and 80,000 times using the Siemens Elmiskop I microscope. The density differences in the original negatives of Figs. 2, 3, and 6 were too great for direct enlargement without complicated "dodging." A positive contrast copy of each negative was therefore made and placed 2 mm above the original negative in the photographic enlarger. When the enlarger lens was focused on the original negative, the positive copy was out of focus, but served as an unsharp mask to "dodge" the picture (35).

RESULTS

ASSAY OF THE ACTIVITY OF THE INHIBITORY MUCOPROTEIN: The hemagglutination inhibition of the inhibitory mucoprotein before and after treatment at 70°C and 80°C respectively, gave the following results: (*a*) complete inhibition of hemagglutination was obtained with concentrations of 5–9 × 10^{-4} μg inhibitory mucoprotein per HA unit of heated A/WS virus. (*b*) Treatment of the inhibitory mucoprotein at 70°C for 45 minutes left a residual activity of about 20 to 25 per cent (*c*) The exposure of the inhibitor to 80°C for 45 minutes left a residual activity of approximately 1 per cent. This residual activity was unchanged after 90 minutes of heating at 80°C (11).

ELECTRON MICROSCOPY: In negatively stained preparations the inhibitory mucoprotein consists of a network of filaments which are bundles of smaller fibrils (Fig. 1). The diameters of the bundles average 120 A but vary between 240 and about 40 A, depending on the number of fibrils (Figs. 1 and 4). Very often smaller bundles or single fibrils split off from one filament and are attached to another filament. In most cases the single fibrils run in "zig-zag" courses[1] with a periodicity of about 110 to 140 A (Figs. 1 and 4). In some cases they seem to be more stretched out and the periodicity is then longer or even obscured. Straight fibrils of the same diameter are seen, usually accompanied by one or more zig-zag fibrils (Figs. 1–4) and running parallel to the main axis of the zig-zags inside or outside of the bundles. It is not clear whether or not they are zig-zag fibrils "stretched" to a straight line form (Fig. 1). The smallest fibrils in our preparations measure about 10 A in diameter.

The measurements of the different fibril-elements are summarized as follows:—

	Diameter	Length
Filament = bundle of fibrils	>240 to 40 A	See text
Single rod-like element of zig-zag measured in the bundles	20 A	55–65 A
Zig-zag fibrils	Apparently 3 groups with element diameters of about 40 A, 20 A, and 10 A	Length of single branch or rod-like element 55 to 66 A; length of zig-zag period 100 to 140 A

[1] The term zig-zag fibril was used to describe the character of this fibril type as seen in the two-dimensional projection. It does not imply any three-dimensional arrangements of the bundles nor does it exclude a helical arrangement.

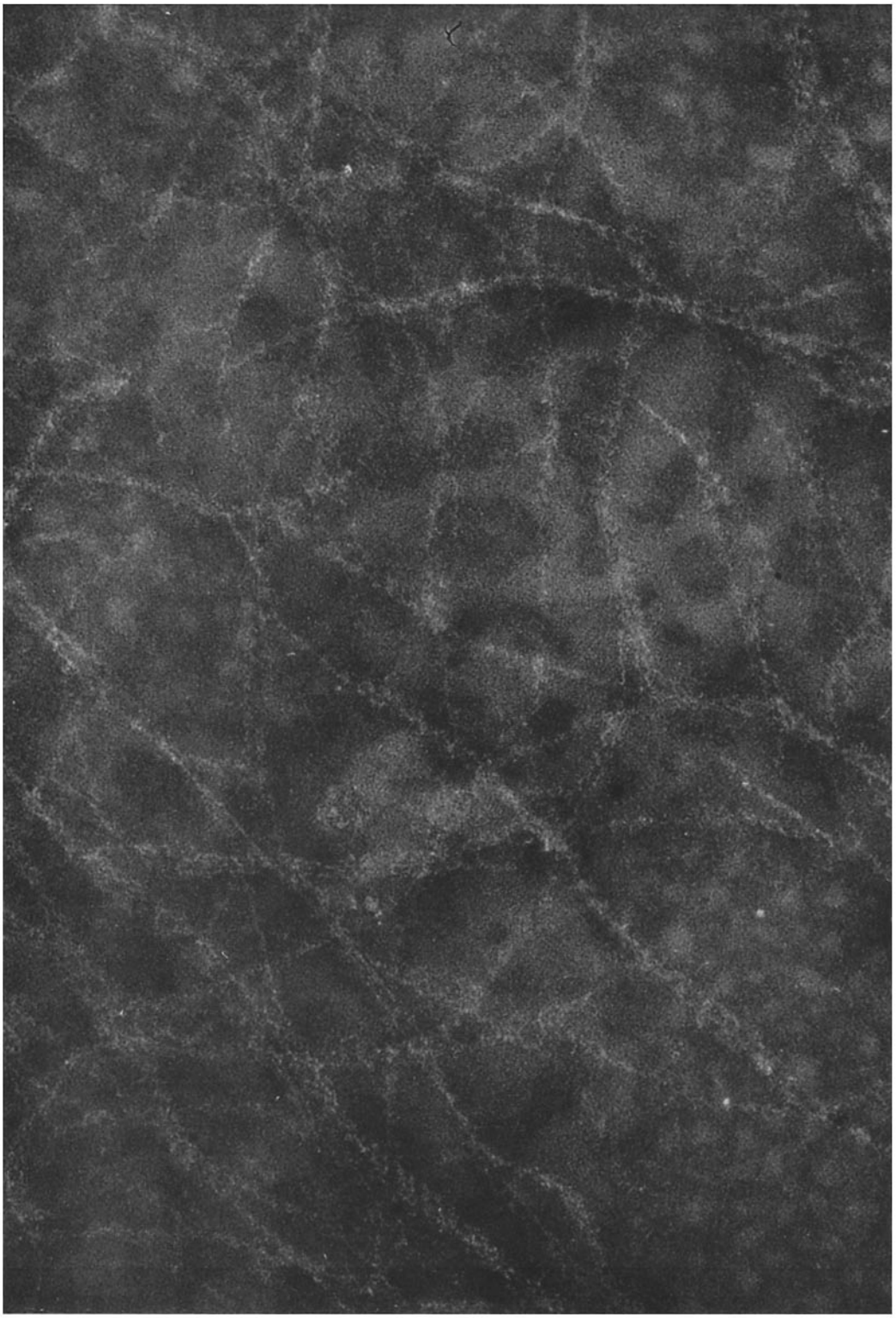

FIGURE 1 Inhibitory mucoprotein filaments negatively stained. × 200,000.

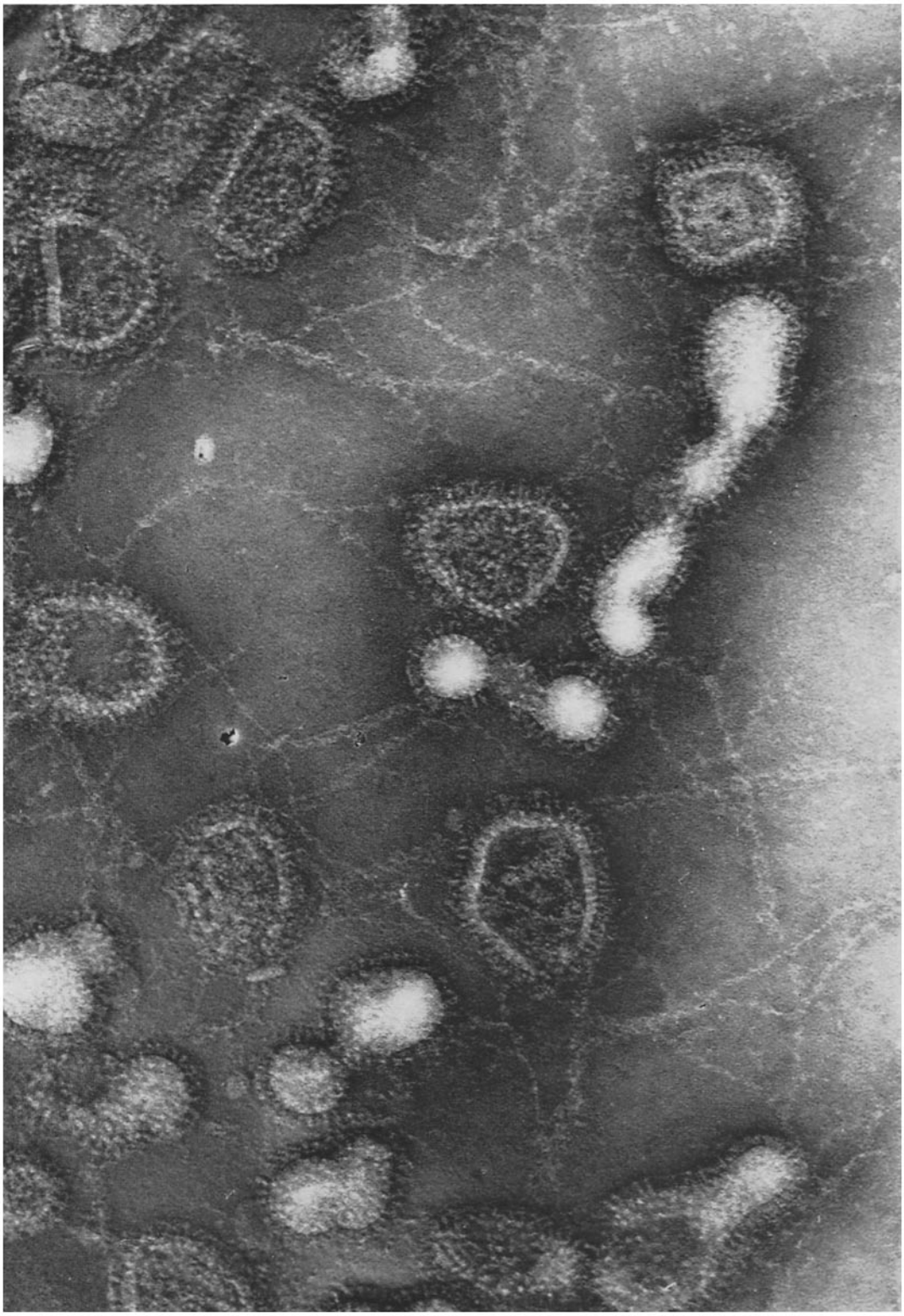

FIGURE 2 Influenza virus particles (A/WS) plus inhibitory mucoprotein. × 200,000.

After the heat treatment at 70°C for 45 minutes, one gets almost the same picture as in unheated material, except that the length of the filaments must decrease because bundle ends are more frequently seen (Fig. 4). However, there was no significant change in the diameters or lengths of the zig-zag elements.

material. Many undefinable fragments are seen, partially in association with the apparently intact rods.

Influenza virus particles are easy to identify by their typical surface projections after negative staining, as shown in Figs. 2 and 3. No fibrils were found in the infected allantoic fluid.

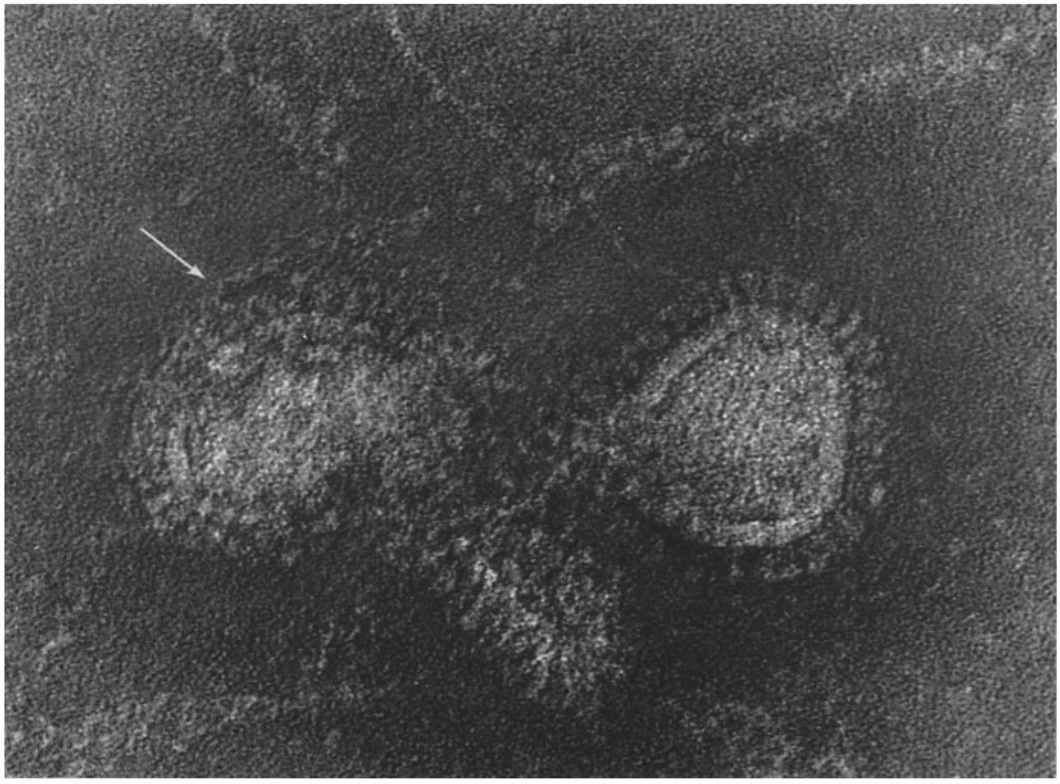

FIGURE 3 Influenza virus (A/Singapore) plus inhibitory mucoprotein; a zig-zag fibril touches the virus projections (arrow). × 380,000.

After an exposure of the mucoprotein to 80°C, the morphology of the material has changed radically. The pictures show many small scattered pieces (Fig. 5) and there is no certain indication of a complete filament. In some places, however, short rods become visible with the dimensions of the zig-zag element; furthermore, at least a few double elements are seen, just forming one V shaped period of the original zig-zag fibril. The diameter of the rods is usually about 20 A. In addition to this, still smaller fibrils with diameters of 10 A or less are seen which might be identical with the smallest zig-zag fibril of the untreated

Exposing influenza virus (A/Singapore and A/WS, respectively) to the inhibitory mucoprotein for some minutes and washing off the supernatant revealed virus particles surrounded by filaments of the inhibitory mucoprotein (Fig. 2). One fibril often touches several projections of the same or of different virus particles. The virus particles are thus connected by this fibril-network. At higher magnifications (Fig. 3) one gets the impression that the corners of the zig-zag fibril are attached to the virus projections. So far, we have seen no differences in the size or pattern of the filaments before and after the virus-inhibitor interaction.

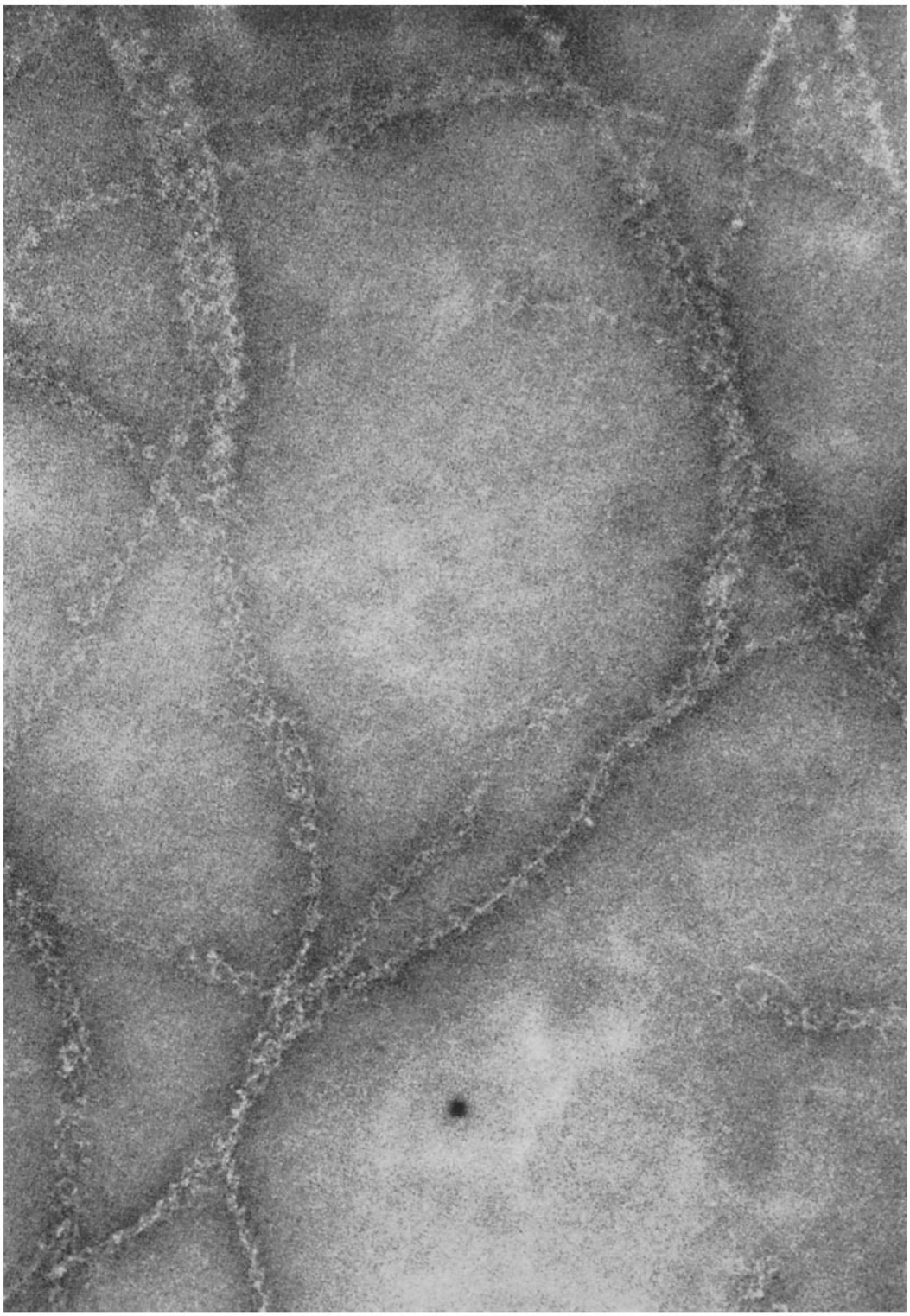

FIGURE 4 Mucoprotein after heating at 70°C for 45 minutes. × 280,000.

The morphological picture of the reaction with mucoprotein heated to 70°C is the same as the picture with untreated inhibitor. On the other hand, fragments of inhibitor heated to 80°C (Fig. 6) do not seem to be adsorbed to the virus surface. The small pieces, which are characteristic for the 80°C material, do not seem to stick to the virus—or they might be less firmly bound and become detached by the change of environment during the negative staining process. However, in a few cases one can still observe some residual larger fibrils, not broken down to short rods, attached to the virus (Fig. 6, arrow).

DISCUSSION

Tamm and Horsfall (11) described the separation of an inhibitor of viral hemagglutination in human urine by precipitation in 0.58 M sodium chloride. They also showed that the portion involved in the inhibition of hemagglutination is soluble in 0.025 M phosphate buffer and can be extracted from frozen-dried mucoprotein. This procedure, which was also used in our experiments, yields a single homogeneous substance, according to electrophoretic (17) and ultracentrifuge studies (18), with a molecular weight of 7×10^6 and an axial ratio of 172. Maxfield (19) confirmed these results and furthermore separated another form of urinary mucoprotein with a molecular weight of 28×10^6 which is supposed to be made up of four molecules with a molecular weight of 7×10^6.

On the other hand, the molecules with the molecular weight of 7×10^6 can be dissociated, by chemical treatment of the mucoprotein (20) or by direct chemical treatment of human urine (21). This yields a smaller molecule, which has a molecular weight of 3.5×10^6; *i.e.*, one-half of the former molecule and with half of its length. This "half" molecule consists of two parts which are slightly different in electrical charge (19) but apparently equal in size or shape. The diameter or width of all of the molecules is considered to be 42 A. Electron micrographs of the urine mucoprotein, published by Porter and Tamm (22), showed long filaments having a width of about 100 A and an average length of 25,000 A. These authors also described a repeating nodose structure of the filaments with a periodicity of about 110 A, which is probably identical with the "zig-zag" periodicity in our preparation. Sharp, Lanni, and Beard (23) also reported the visibility of linear aggregates of spheroidal particles with a width of 50 to 100 A in the egg white inhibitor.

With respect to the length of the filaments, one finds a great variation in the reports. Porter and Tamm (22) reported filaments measuring 2500 A to 40,000 A in length, the majority being greater than 15,000 A. Among fibrils of variable lengths, Maxfield (19) frequently found fibrils with a length of 12,000 A and a diameter of less than 100 A and some smaller fibrils with a width less than 50 A; he considered the molecule to be ribbon-shaped.

All the authors mentioned above used the shadow casting method, which did not permit as good resolution of the fine structure of the molecules as can be obtained with the negative stain technique, invented by Hall (13), Huxley (14), and Horne and Brenner (15). A similar example of increasing the practical resolution of the electron micrographs by using negative staining is the demonstration of the isolated actin filaments by Hanson and Lowy (24). These authors showed two helically wound strands composed of spherical subunits forming the filament, whereas with the shadow casting method, in spite of high technical performance, Rozsa, Szent-Györgyi, and Wyckoff (25) could not see the detailed structure.

In negatively stained preparations of the mucoid inhibitor we could not determine the length of the filaments because of their tendency to form end-to-end aggregates, a characteristic behavior in the more concentrated preparations (Maxfield, reference 19). However, the other dimensions of the filaments and fibrils were definable.

The splitting of the bundles and/or the association with other bundles, thus leading to a network formation (Fig. 1), results in a wide range of bundle diameters from 240 A down to 40 A; the bundles all seem to follow the same principles of architecture, and the only morphologic differences are in the number of fibrils composing the filaments. A bundle diameter of 120 A, however, occurs with relatively high frequency, in the untreated as well as in the heat-treated preparations. The most prominent aspect of the fibrils is the frequent occurrence of the zig-zag configuration.

The fine structure of the bundles reveals a

complicated pattern, which is probably made up by the association of single "zig-zag" fibrils and, in some cases, also of additional straight fibrils. In most of the pictures, the bundle and the small zig-zag fibril predominates. The term "zig-zag" fibril used to describe the two-dimensional picture of a single-lying fibril does not imply a two-dimensional make-up of this fibril nor exclude a coiling of the fibrils. The pictures cannot show unequivocal evidence for a definite type of three-dimensional arrangement, but a helix seems to be the most probable configuration. A reconstruction of a model fibril from the electron micrographs cannot be satisfactory because of the variable angle between two single branches of a zig-zag as the result of stretching and other distortions produced by the preparative method.

A picture of a "zig-zag" fibril can easily be obtained by looking at a two-dimensional projection of a helix; in that case the corners of the zig-zag should be rounded. Unfortunately, the electron microscope does not permit us to detect this detail in spite of high resolution; it would still show a rounded corner independently of the actual shape of that small portion of the molecule, a problem which has been discussed in general by von Borries and Kausche (26).

Gibbons and Glover (27) interpreted the anomalous birefringence of bovine cervical glycoproteins as indicating molecules of random coil form. X-Ray diffraction patterns (28) on bovine submaxillary gland mucoprotein both as a powder and as a stretched film indicate that the mucin is amorphous. Gottschalk and McKenzie (29) reported x-ray studies on ovine submaxillary gland mucoprotein which revealed a rubber-like pattern which consisted of two ill-defined amorphous rings in the range of 4 to 20 A. When examined at low angles, the first and second orders of a spacing of about 100 A were detected. Infrared studies (28) showed some indication that the protein is in a β-configuration or other ex-

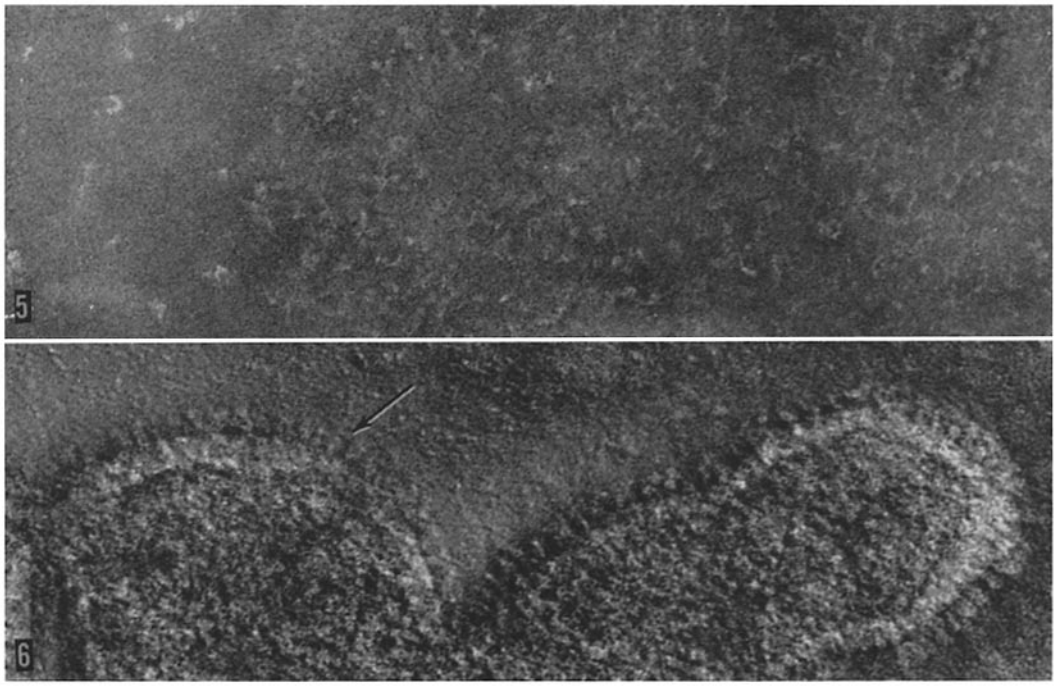

FIGURE 5 Mucoprotein after heat treatment at 80°C for 45 minutes. × 280,000.

FIGURE 6 Mucoprotein heat-treated at 80°C for 45 minutes, added to influenza virus particles (A/WS). At one point (arrow) a small fibril is attached to the virus surface. × 280,000.

tended form and not an α-helix. One can assume that the 100 A spacing corresponds to the 100 A periodicity seen in the electron microscope by Porter and Tamm (22) which is caused by the zig-zag unit seen in our pictures in connection with the fibril bundle.

The measurement of the diameter of the elementary fibrils shows at least 3 groups: 40 A, 20 A, and 10 A or less. The width of 40 A is in accordance with ultracentrifugal and electrophoretical studies of Tamm, Bugher, and Horsfall (18) and Maxfield (19). The latter author (1963) showed, in a study with ultracentrifugation and electrophoresis, that the molecules can be split transversely into small fragments. Shadow casting in his electron micrographs did not permit the study of the detailed structure of the rod-like molecules.

Degeneration after heating to 70°C is demonstrated in the decrease of the inhibitory activity (12), in spite of the existence of shorter bundles which should have a higher activity because of an increased collision frequency. The fragmentation of the molecules after heating was shown, by Tamm, Bugher, and Horsfall (18), in a marked reduction of viscosity and a more slowly migrating diffuse boundary in the ultracentrifuge. It is also indicated by the increasing number of free-ending filaments in electron micrographs (Fig. 3). An almost complete destruction of the filamentous molecule is seen in the micrographs after a heat treatment at 80°C for 45 minutes, which yields almost exclusively small rod-like pieces, "zig-zags", and round or fuzzy fragments. This material had lost 98 per cent of its inhibitory activity in the experiments of Tamm and Horsfall (11), the same order of magnitude that we found.

The morphology of the interaction between influenza virus particles and the mucoprotein inhibitor is shown in Figs. 2 and 3; in some aspects it is similar to that shown in the schematic drawings of Buzzell and Hanig (30). In most cases more than one filament is attached to the surface of one virus (Fig. 2). At the concentration of inhibitor used here, usually more than one virus particle is attached to one filament, thus revealing a wide-meshed network with virus particles at the junction points.

The virus surface with its typical projections is seen to be touched by the corners of the zig-zag (Fig. 3). Assuming a coiled inhibitor-fiber, this would simply indicate that the outermost part of the fibril is involved in the adsorption. The attachment of virus particles to the receptor surface of red blood cells, which is considered to be a similar process, happens also on the outermost surface of the erythrocyte, on a relatively large plane of adsorption, as shown in ultrathin sections (31).

In other fields of the preparations one might have the impression of a deeper penetration of the fibers into the surface patterns of the virus particles (Fig. 2). Those cases could be explained either by an overlapping of projections of the virus surfaces in the preparation or by the assumption that the interaction of virus and fiber also takes place at sites of the virus surface other than the tips of the projections.

Influenza virus destroys the inhibitory activity with its receptor destroying enzyme after exposure to the mucoprotein substrate. However, the action on the substrate along the macromolecular mucoid (Gottschalk, 32, 33), which leads to a decrease in the electrophoretic mobility of the inhibitor by 20 per cent (17), is not visible in the micrographs; in other words, no specific morphological change on the molecule has been detected that could be attributed to the adsorption and subsequent elution of a virus particle.

Experimental data of Fazekas de St. Groth (34) fit very well into the morphological picture of virus-inhibition by indicating that the reactive sites on the virus surface are not accessible to subsequently added antibody as long as they are "shaded" by the bulky inhibitor molecule.

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