Molecular Properties of Rous Chicken Sarcoma
Hyaluronic Acid-Protein Complex

A. CAPUTO AND M. L. MARCANTE

("Regina Elena" Institute for Cancer Research, Rome, Italy)

Hyaluronic acid (HA), first isolated by Meyer (1), is a compound of considerable biological interest. This polysaccharide has been found in many animal tissues, especially those of mesenchymal origin, and in the capsules of some types of bacteria.

Furthermore, a mucinous polysaccharide of the HA type was detected and isolated from fowl sarcoma by Kabat (10) and by Claude (5). These findings have been confirmed recently by Warren and co-workers (17), by Caputo (3), and by Harris, Malmgren, and Sylvén (9).

In the course of some research related to the importance of mucoproteins in tumor growth, we became interested in the isolation and characterization of Rous chicken sarcoma HA.

In this paper will be described the results of a study on the physicochemical properties of HA isolated from Rous chicken sarcoma, comparing it with the same compound prepared from normal sources, such as cock's comb, ox synovial fluid, and human umbilical cord.

MATERIALS AND METHODS

Tumors were collected from 2–3-month-old chickens. The material, freed of necrotic areas and reduced to small pieces, was defatted with cold acetone containing a few drops of acetic acid; the acid acetone was twice replaced with cold acetone. After 48 hours the pieces were dried and then homogenized in a Waring Blendor with 10 volumes of distilled water. The pH was then brought to 9 by adding a few drops of 0.1 N NaOH.

Extraction was carried out for 48 hours with occasional stirring. The mixture was filtered through cheesecloth, and the centrifugally cleared extract was acidified to pH 4.0 with 0.1 N HCl. The salt concentration of the extract was brought to 0.2 M by adding NaCl, and the mucopolysaccharide was then precipitated by addition of 3 volumes of acetone. The precipitate, collected with a stirring rod, was redissolved with distilled water, dialyzed against distilled water until salt-free, and then lyophilized. All manipulations were performed in the cold room at +2 °C.

In the case of ox synovial fluid after defatting, the pH was brought to 4.0, and then precipitation of HA complex was obtained, as described above, by adding NaCl and 2 volumes of cold acetone.

From chemical analysis it is apparent that HA prepared by this method must be considered as an HA protein complex. Quantitative estimations of protein by means of the biuret reaction (8) have shown that the HA from Rous chicken sarcoma contains about 15 per cent of protein and the HA's from umbilical cord, ox synovial fluid, and cock's comb contain, respectively, 10, 13, and 20 per cent of protein.

None of the preparations contained heparin, as ascertained by the clot-forming method. The absence of phosphorus and of a specific absorption in the U.V. region both indicate that the HA-protein complex does not contain nucleic acids.

Electrophoretic experiments were performed with moving boundary Perkin-Elmer apparatus, and mobilities (μ × 10^4 sq. cm. sec^-1 volt^-1) were calculated as means of the values of the ascending and descending limbs. All the runs were made with buffer systems according to Miller and Golder (18).

The sedimentation studies were made in the Spinco electrically driven ultracentrifuge at 50,780 r.p.m. The values of the sedimentation constant, corrected for viscosity and density of water to 20 °C., are given in Svedberg units (S_n,20 × 10^-13).

Diffusion measurements were made at +1 °C. in the electrophoresis separation cell according to the height-area method. For sedimentation and diffusion constant measurements, the media viscosities were determined on a Ostwald capillary viscosimeter with a water flow time of 4.24 sec. The densities of the media were calculated in a specific gravity pipette.

RESULTS

The electrophoretic behavior of Rous sarcoma HA-protein complex is characterized by the presence of one very sharp single boundary, which migrates rapidly with a mobility, at pH 7.0, of μ = 14.5 × 10^-5. For HA-protein complex isolated from sources different from Rous sarcoma, such as cock's comb, human umbilical cord, and ox synovial fluid, we have obtained the same electrophoretic pattern.

In the ultracentrifuge, the Rous sarcoma HA-protein complex sediments as a monodisperse system, as indicated by the presence of one single very sharp boundary at both higher and lower concentrations. The same characteristics have been found for the compounds prepared from other sources; the sedimentation diagrams of Rous sarcoma and cock's comb HA-protein complex are shown in Chart 1.
The sedimentation constant of HA-protein complex depends to a considerable extent upon its viscosity, and therefore the values are closely related to the concentrations, as is shown in Chart 2, where the sedimentation constant, corrected for temperature and viscosity of medium, is extrapolated to 0 concentration.

We have studied the stability of our preparations in the ultracentrifuge as a function of pH: as shown in Chart 3 the behavior of tumoral HA-protein complex closely resembles that of HA from different sources. The pH stability region is confined to the zone pH 5.0 and 8.0, above and below which the sedimentation constants at infinite dilution decrease. HA precipitates from solution below pH 3.0.

In order to ascertain the influence of the medium on the stability of HA-protein complex a few experiments were performed with high salt concentrations. Changes were observed in the ultracentrifuge at the highest salt concentrations (Chart 4).

In the diffusion experiments each run was performed in duplicate, and each half of the cell was filled with a different sample of HA. The photographs were taken at intervals of between 70 and 120 hours, and the values averaged. In Table 1 are reported the values obtained for HA-protein complex of different sources.

The partial specific volume has been measured in 5-ml. pyknometers by plotting the weights of the contents of the pyknometers for several concentrations of HA-protein complex against the concentration expressed as a weight fraction. The same mean value of \( \bar{V} = 0.703 \) has been obtained, without appreciable differences, for HA-protein complex of each source. The molecular weight has been calculated using the usual formula of Svedberg, where \( M = Rts/D(1 - \bar{V}_p) \), and substitut-
DISCUSSION

Evidence has been obtained to show that HA is present in appreciable quantities in Rous chicken sarcoma, thus confirming the reports of previous findings (Kabat [10], Claude [5], Warren and co-workers [17], Caputo [3], Harris and co-workers [9]). HA has been isolated by a method which represents a slight modification of the old procedure of acetone precipitation.

The mucopolysaccharide obtained from Rous sarcoma and other sources by this procedure must be considered as HA-protein complex and is a relatively pure substance as judged from some of its physicochemical properties. Thus, it behaves in the electrophoresis as a monodisperse compound exhibiting one single, sharp boundary with a high mobility. The homogeneity of HA-protein complex has been confirmed by the ultracentrifuge experiments, in which in the sedimentation diagram is present one single, very sharp boundary.

A variation in molecular weight has been found between HA-protein complex from the tumor and that from normal sources: for the first a value of 760,000 has been found, while for the others the molecular weight is of the order of 1 million.

In a comparison of our results with those reported by previous workers (1, 4, 6, 11, 14-16), it will be noted that the discrepancies are not significant when the molecular weight data were obtained with the same procedure. The only difference found was in the partial specific volume, which was slightly different from those reported by Ogston and Stanier (14) and by Varga (16). The value we have found falls within the range of many other polysaccharides, and the discrepancies could be attributed to the different percentages of protein present in our preparations.

To ascertain whether the difference in molecular weight is due to the degree of polymerization or to a peculiar arrangement of the molecule, we have studied some other physicochemical properties,

<table>
<thead>
<tr>
<th>Source</th>
<th>Concentration (g/ml)</th>
<th>(D_{20} \times 10^{-7})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rous sarcoma (chicken)</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Comb (cock)</td>
<td>0.5</td>
<td>1.0</td>
</tr>
<tr>
<td>Umbilical cord (human)</td>
<td>0.4</td>
<td>0.9</td>
</tr>
<tr>
<td>Synovial fluid (ox)</td>
<td>0.3</td>
<td>1.1</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Source</th>
<th>(M_w \times 10^6)</th>
<th>(t/t^*)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rous sarcoma (chicken)</td>
<td>0.76</td>
<td>2.75</td>
</tr>
<tr>
<td>Comb (cock)</td>
<td>1.01</td>
<td>2.65</td>
</tr>
<tr>
<td>Umbilical cord (human)</td>
<td>1.05</td>
<td>2.65</td>
</tr>
<tr>
<td>Synovial fluid (ox)</td>
<td>1.07</td>
<td>2.60</td>
</tr>
</tbody>
</table>
fect is evident from the computed sedimentation constants.

Evidence has been presented in this paper that HA occurs in Rous sarcoma in a molecular state slightly different from that isolated from normal sources. If we consider that the tumor HA-protein complex has physicochemical properties similar to the HA isolated from normal sources, with the exception of a lower molecular weight, it might be concluded that the difference is due to a smaller degree of polymerization.

Now the following question arises: is the tumor HA synthesized in the same way as in a normal tissue, or is a depolymerizing system present in the tumor which is able to act on the usually formed HA? It seems unlikely to admit that the synthesis of HA in tumor might follow steps different from those normally observed.

For this purpose we have performed a few experiments, in which it has been observed that the incubation of synovial fluid HA with the tumor extract, deprived of its HA, was able to reduce the molecular weight of synovial fluid HA from 1.07 to 0.80 × 10^6. It seems, therefore, according to Gersh and Catchpole (7), that the tumor contains an enzymatic system which depolymerizes HA. Preliminary observations on the mechanism of action of this depolymerizing system have shown that it is almost different from the well known hyaluronidase, and perhaps some enzyme of the proteolytic type could play a great role in the degradation reaction. Studies on the isolation and characterization of the HA depolymerizing system present in the Rous sarcoma are in progress, and the results will be reported in a forthcoming paper.

SUMMARY

The molecular properties of HA-protein complex isolated from Rous chicken sarcoma have been studied. It has been shown that tumoral HA-protein complex behaves in electrophoresis and in the ultracentrifuge as a monodisperse system.

The molecular weight of tumor HA-protein complex is 760,000, while that isolated from normal sources, such as cock’s comb, ox synovial fluid, and human umbilical cord, has a molecular weight of the order of 1 million.

REFERENCES
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