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Properties of Rous Sarcoma Hyaluronic Acid.* (29923)

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Morphological and biochemical studies(1, 2) of connective tissue in cancer have led to the view that the invasive growth of malignant tumor may be due to destruction of the ground substances of connective tissues adjacent to the tumor or to proliferation of undifferentiated connective tissues. Little is known concerning the biochemical characteristics of cancerous connective tissue components; however several workers(3,5) have indicated that the Rous sarcoma hyaluronic acid or its protein complex has a lower particle size than that of normal tissue as determined by viscosity and sedimentation constant.

In the work described here, the hyaluronic acid of Rous sarcoma was found to behave differently from that of normal tissues in the differential precipitation procedure with a cation detergent(6,7) and in ECTEOLA cellulose anion exchange column chromatography(8). The hyaluronic acid preparation from the sarcoma was also found to increase the rate of spreading of trypan blue in skin (9) and to increase capillary permeability (10).

Experimental. Sources of hyaluronic acid. Rous sarcoma was grown subcutaneously under the wings of white Leghorn chickens. Forty or fifty days after inoculation of Rous virus, the rapidly growing tumors were collected as a source for preparation of mucopolysaccharide. The necrotic brei, if any, in the central part of the tumor was discarded. Necrotic areas, when present, could easily be distinguished from viable tissue by the darker color and comparative softness. After the necrotic material was removed together with adjacent intact tumor tissue, the remaining tissue was washed with tap water before proceeding with preparation of mucopolysaccharides. Bovine vitreous humor and human umbilical cord were used as a source of normal hyaluronic acid. All tissues were defatted with acetone, dried and then ground in a Wiley laboratory mill.

Preparation of crude mucopolysaccharide. The tissue powder was digested with pepsin and then with trypsin according to the method of Jeanloz *et al*(11) and Schiller *et al* (12). Perchloric acid was added to precipitate the residual protein; the supernatant was dialyzed against distilled water and then lyophilized. The uronic acid content of isolated crude mucopolysaccharide preparation, estimated with the carbazole reaction of Dische(13), was as follows: Rous sarcoma, 26.5%; vitreous humor, 38.8%; umbilical cord, 35.9%.

Preparation of alkali-degraded hyaluronic acid. The mucopolysaccharide preparation of vitreous humor and of umbilical cord was dissolved in 1 M NaOH and incubated at 37° for 48 hours. After neutralizing, the solution was dialyzed against distilled water for 48 hours.

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Preparation of hyaluronidase degraded hyaluronic acid. One ml of 0.1% mucopolysaccharide preparation in 0.3 M phosphate buffer of pH 5.5 was mixed with 1 ml of 0.05% hyaluronidase (300 USP U/mg, Nutritional Biochemicals Corp.) and incubated at 37° for 15 or 60 minutes. This was followed by addition of one-third volume of 17% perchloric acid in the cold; the precipitate was removed and the supernatant was dialyzed against distilled water for 72 hours.

Precipitation reaction with cetyltrimethylammonium bromide (CTAB). This procedure was carried out as described by Scott (6) and Schiller *et al*(7), using 0.1% CTAB or cetylpyridinium chloride (CPC) in 0.04 M NaCl solution. The amount of recovered hyaluronic acid was estimated by the uronic acid determination. By this procedure, the recovery of Rous sarcoma hyaluronic acid was only 60-70% of the original. However, the yield increased upon addition of alkaline solution (Table I), reaching as high as 96% at 0.1 or 0.2 M NaOH. Only CTAB can be used for the alkali-precipitation reaction since CPC is unstable in alkaline solution. Therefore, for the comparative CTAB-precipitation studies of various hyaluronic acid preparations, the mucopolysaccharide was precipitated by 0.1% CTAB in 0.04 M NaCl sclution. After centrifugation, the clear supernatant was removed, one-tenth volume of 1 M NaOH was added to it, and the mixture was left overnight at room temperature. The resulting precipitant (alkali-precipitable fraction) was obtained by centrifugation after adding a heavy celite. The hyaluronic acid from both fractions was obtained by eluting with 0.4 M NaCl, and uronic acid content was determined with the carbazole reaction on all samples.

ECTEOLA anion exchange column chromatography. ECTEOLA cellulose (capacity: 0.3 meq/g, Carl Schleicher & Schuell Co.) was washed with 0.5 M NaOH, then with 3 M NaCl and finally with 0.1 M NaCl-HCl mixture (1 : 1) after the method of Ringertz and Reichard(8). The washed exchanger was suspended in a 1:1 mixture of 0.1 M NaCl and 0.1 M Na acetate buffer, pH 5.0, and packed into a column 1 cm wide and 20 cm long. Fifteen mg of crude mucopolysaccharide were dissolved in 10 ml of the above suspending solution and applied to the column. Elution was carried out step-wise with 0.05-0.8 M NaCl in 0.05 M Na acetate buffer, pH 5.0. One hundred ml of eluting solution were used in each step and 16.0-16.5 ml fractions were collected with an automatic fraction collector. The rate of elution was 210-230 minutes per 100 ml. The uronic acid content in each effluent was determined by the carbazole method, and the impurities such as protein and nucleic acid were detected by measuring the absorption at 260 mμ.

Chemical analysis of fractions of ECTEOLA column chromatography. Hexosamine content was estimated by the Boas modification(14) of the Elson-Morgan reaction. Glucosamine and galactosamine were identified by the method of Stoffyn and Jeanloz(15). Non-hexosamine neutral carbohydrate was determined by the tryptophan method of Shetlar *et al*(16). Protein content was measured by the method of Lowry *et al*(17) and ester sulfate by that of Egami *et al*(18). The reducing power (terminal glucose equivalent(19)) was determined by the modified ferricyanide method of Park and Johnson(20).

Biological assay as spreading factors(9). The hyaluronic acid fractions obtained from the ECTEOLA column chromatography were pooled, dialyzed against distilled water and lyophilized. An amount such that the uronic acid content was 1% was dissolved in 0.85% NaCl solution. As a comparison, hyaluronidase in saline (6,000 USP U/ml) was used. A mixture of 0.1 ml or 1% trypan blue in saline and 0.1 ml of test solution or saline as a control was injected intracutaneously into the skin of the back of a rabbit. One hour later, the large (D) and small (d) diameters of the colored area were measured and its ellipsoid area was estimated by the equation: area $= \pi \times D \times d/4$.

Capillary permeability promoting test. One-tenth ml of test solution or saline was injected intracutaneously into the skin on the back of a rabbit. It was immediately followed by injection of 10 ml of one per cent trypan blue in saline into the ear vein of the same animal. At 15 minutes after injection of dye, the accumulation of dye into the in-

TABLE I. Recovery of Hyaluronic Acid from Various Sources from Cetyl Trimethylammonium Bromide-Precipitation in Neutral and Alkaline Solutions.

		Precipitable in neutral		Precipitable in alkali*	
	Added	Found	% recovered	Found	% recovered
		(μg of uronic acid)			
Rous sarcoma	509	338	66.4	148	29.1
Vitreous humor					
Intact	801	764	95.4	13	1.6
Alkali-degraded†	801	554	69.2	100	12.5
HAase-treated‡					
15 min	388	180	46.4	136	35.1
30 "	388	96	24.7	168	43.3
60 "	388	24	6.2	144	37.1
Umbilical cord					
Intact	1036	1016	98.0	9	.9
Alkali-degraded†	1036	652	62.9	122	11.8
HAase-treated‡					
15 min	359	250	70.0	78	21.7
30 "	359	144	40.1	138	37.9
60 "	359	78	21.7	152	42.4

* 0.1 M NaOH solution.
† Incubated in 1 M NaOH solution at 37° for 48 hours, neutralized and dialyzed.
‡ Incubated with testicular hyaluronidase at 37° for time indicated.

jected skin area was estimated by measuring the colored area as above.

Results. Table I shows the recovery of various hyaluronic acid samples in the CTAB precipitations in neutral and alkali solutions. The hyaluronic acid of vitreous humor and umbilical cord was almost completely recovered in the fraction precipitable at neutrality. The recovery of partially hydrolyzed hyaluronic acid in the neutral-precipitable fraction was lower than that found in untreated samples, and the alkali-precipitable fraction of the hydrolyzed samples was correspondingly higher. The hyaluronic acid of Rous sarcoma exhibited a recovery pattern similar to that of the partially hydrolyzed one. In the case of partially hydrolyzed hyaluronic acid, the total recovery was lower than the original, possibly because some of it was depolymerized too extensively to precipitate even in an alkaline solution.

Fig. 1 shows the ECTEOLA anion exchange column chromatograms of various hyaluronic acid preparations. The hyaluronic acid of Rous sarcoma and the hyaluronidase-treated hyaluronic acid of vitreous humor were largely eluted at 0.2 M NaCl fraction. On the other hand, the intact hyaluronic acid of vitreous humor and umbilical cord was present only in traces in this fraction, but was found mostly in 0.4 and 0.6 M NaCl fractions. The impurities which have an absorption at 260 mμ were largely eluted in 0.05 M NaCl fraction and were found in only small amounts in the fractions of higher NaCl concentration.

Results of the chemical analysis of each fraction of ECTEOLA column are shown in Table II. The molar ratio of hexosamine to uronic acid was nearly equal to unity in all fractions. The absorption curve, after the tryptophan reaction, did not show a peak at 500 mμ, indicating that neutral sugars such as galactose and mannose were not present. Only glucosamine was found as the hexosamine constituent by paper chromatography. Only a slight amount of ester sulfate was in the 0.6 M NaCl fraction of umbilical cord preparation. Sulfate was not found in other fractions. These results indicate that the mucopolysaccharide of these fractions (0.2-0.6 M NaCl fractions) is composed almost exclusively of hyaluronic acid. The protein content was higher in Rous sarcoma preparations than in the others.

The ratio of uronic acid to terminal glucose equivalent, which may be an indicator of molecular weight, was much lower in the

TABLE II. Analysis of Hyaluronic Acid Fractions from ECTEOLA Column.

	Vitreous humor		HAase-treated vitreous humor*		Umbilical cord			Rous sarcoma	
Molarity of eluting NaCl	.4	.6	.4	.6	.2	.4	.6	.2	.4
Recovery, %	56	25	43	20	6	37	20	51	28
Molar ratio of glucosamine to uronic acid	.97	.94			.94	.95	.96	1.01	1.06
Protein/uronic acid, %	1.3	2.3			2.6	3.6	2.3	12.1	4.0
Ratio, g/g, of uronic acid to terminal glucose equivalent	42.1	49.0	15.0	16.6	39.6	57.7	55.0	4.7	7.9
CTAB precipitable									
neutral, %	95.3	97.3	94.1	95.5	89.6	96.9	94.7	12.6	82.0
alkali, %	1.5	0	1.3	.5	5.2	1.8	2.0	82.5	15.2

* 30 minutes incubation with hyaluronidase.

preparation from Rous sarcoma. This ratio was lower in the fraction of 0.2 M NaCl than that of 0.4 M. The hyaluronidase-treated hyaluronic acid also had a lower ratio than the intact hyaluronic acid, but it was higher than the Rous sarcoma hyaluronic acid.

In the CTAB precipitation reaction, all fractions of vitreous humor and umbilical cord were precipitable in neutral solution; those of Rous sarcoma were only partially precipitable. The 0.2 M fraction from this tumor was mostly precipitable in alkaline solution. In the case of hyaluronidase-treated hyaluronic acid, both 0.2 and 0.4 M fractions were precipitable in neutral solution in contrast to those of Rous sarcoma. As the eluted fractions were dialyzed in the presence of neutral salt before the precipitation reaction, it seems probable that the material of smaller particle size, which might be precipitable with CTAB in alkalinity, was dialyzed out. The results of the spreading and capillary permeability promotion test (Fig. 2) demonstrated that Rous sarcoma hyaluronic acid had positive activities in both tests, in contrast to the negative reaction of the normal hyaluronic acid.

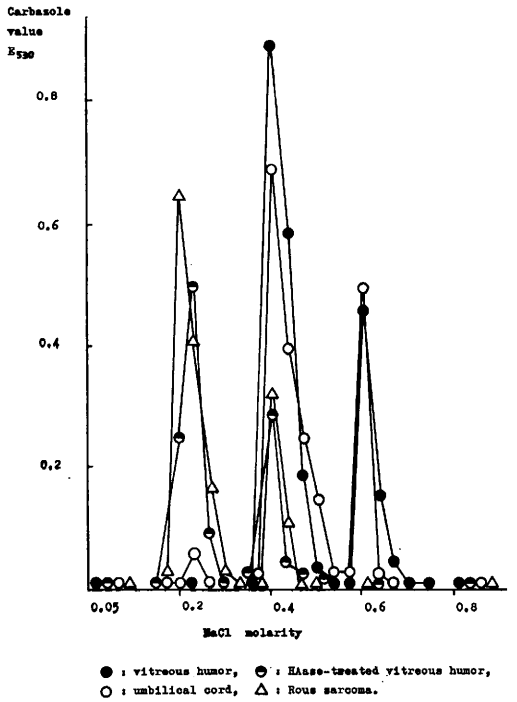

FIG. 1. Eluting patterns of various hyaluronic acid samples from ECTEOLA column.

Discussion. Rous sarcoma hyaluronic acid was not completely precipitated with cation detergent in the dilute saline solution in which the hyaluronic acid from normal tissue precipitated completely, and it was eluted at lower NaCl concentration than the normal from the ECTEOLA anion exchange column. Hyaluronic acid, partially depolymerized with hyaluronidase or alkali treatment, tended to have the same character as the Rous sarcoma hyaluronic acid. The difference between Rous sarcoma hyaluronic acid and other normal hyaluronic acids may, therefore, be due to the lower molecular size of the former.

From the viewpoint that the normal acid mucopolysaccharide production forms the necessary environment for formation of connective tissue fiber(21), the hyaluronic acid found in the Rous sarcoma might lead to

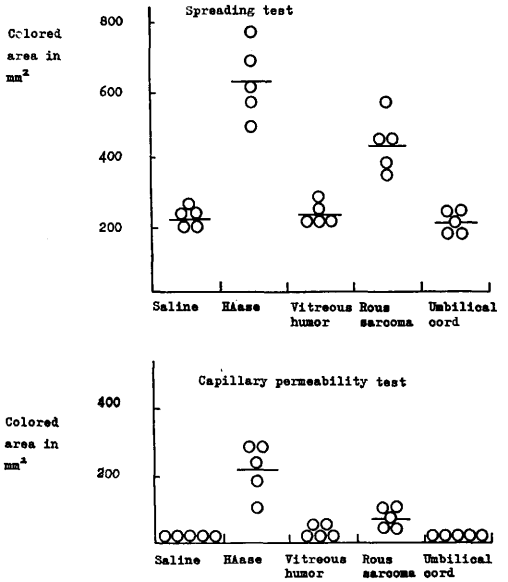

FIG. 2. Horizontal bar shows mean value in each column.

failure in producing mature fibers and, therefore, influence the structure of the tumor. If enzymes which result in this condition are elaborated into the tissue adjacent to the tumor, similar connective tissue, which might allow invasion, could result. Related to invasive ability, it was found that the Rous sarcoma hyaluronic acid has a positive spreading reaction similar to hyaluronic acid, partially depolymerized with hyaluronidase as reported by Seifter and Baeder(9). The strong capillary permeability promoting action of Rous sarcoma hyaluronic acid may be one of the chemical factors accounting for the strong affinity of cancerous connective tissue stroma for intravenously injected Evans blue, as reported by Gersh and Catchpole(10). At present, however, the presence of a factor other than hyaluronic acid in the Rous sarcoma cannot be ruled out.

Summary. Hyaluronic acid isolated from Rous sarcoma was found to have an unusual effect on the precipitation reaction with cation detergent and ECTEOLA column chromatography, and to have an apparently smaller particle size than hyaluronic acid from normal tissues. This preparation exhibited promoting effects upon the spreading of a dye in tissue and upon capillary permeability. A possible role in the invasion properties of tumors is indicated.

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