

Hyaluronidase Activity of Normal and Neoplastic Interstitial Fluid¹ (34568)

B. FISZER-SZAFARZ² AND P. M. GULLINO
(Introduced by W. E. Heston)

*Laboratory of Biochemistry, National Cancer Institute, National Institutes of Health,
Department of Health, Education, and Welfare, Bethesda, Maryland 20014*

In a previous work it was observed that the hyaluronic acid content of the interstitial fluid (IF) of Walker carcinoma 256 was constantly less than in the subcutaneous area where the tumor was transplanted (1). Moreover, small molecular weight hyaluronic acid was found to act as competitive inhibitor of the hyaluronidase activity in serum of tumor-bearing patients (2). An increase of hyaluronidase activity by the tumor could explain both findings, but this has not been demonstrated (3). In the work presented here the interstitial fluid (IF) of Walker carcinoma was sampled, and the hyaluronidase activity was measured and compared with the activity of the IF of the subcutaneous area where the tumor was transplanted. It was found that the hyaluronidase activity was about twice as high in the IF of the tumor as compared with the normal subcutaneous area, and in the serum of the tumor-bearing host the hyaluronidase activity was reduced to one half the level found in normal rats.

Materials and Methods. Male Sprague-Dawley rats, 3 months old, were used. The animals were starved overnight, and blood or IF was sampled in the morning. Blood was withdrawn from the abdominal aorta with plastic syringes after the animal was rendered unconscious by a blow in the occipital region. Serum was prepared by leaving the blood at +5° for 3–4 hr followed by centrifugation at +5°. The IF of the tumor was sampled with a chamber formed by Millipore

filters³ of 0.45- μ pore diameter and incorporated by the tumor as previously reported (4). The chamber was placed in a pouch of the subcutaneous tissue of the scapular region, and the IF collected in the chamber was sampled several days later. At sampling time 2 drops of IF were placed into thioglycollate broth and the culture was kept at 37° for 10 days to test the sterility of IF.

Three different experiments were performed. In each experiment two groups of 10 animals were used. One untreated group furnished the normal blood serum. The second group received, first, a micropore chamber in the scapular region (4) from which IF was sampled after 6 days, then fragments of Walker carcinoma 256 were transplanted around the same chamber, and the IF was sampled after 7–9 days when the tumor reached a size of 5–10 g and had engulfed the chamber. At this time the animal was killed, and the blood serum was taken. Three samples were therefore analyzed from the same animal, and the IF of normal and neoplastic tissue was sampled in the same area by the same micropore chamber.

The hyaluronidase activity present in different fluids was measured by the release of polysaccharide fragments having terminal *N*-acetylglucosamine (NAGA) from a known solution of hyaluronic acid incubated at pH 3.5. To 150 μ l of IF 1.5 ml of hyaluronic acid in formate buffer, pH 3.5, were added (1.0 mg/ml of hyaluronic acid in 0.3 *M* NaCl–0.05 *M* formate buffer). The mixture was incubated at 37°, and aliquots were removed at 0, 0.5, 1.0, 2.0, and 4.0 hr for the

¹ The technical assistance of F. H. Grantham and D. Hill is gratefully acknowledged.

² On leave from: Institut du Radium, Batiment 110, 91 Orsay, France (present address).

³ Millipore Filters Company, Bedford, Massachusetts.

TABLE I. Polysaccharides Fragments with Terminal *N*-Acetylglucosamine Liberated.^a

	NAGA	
	(μ moles/ml fluid)	% Total NAGA ^b
Normal serum	1.05	39.8
Serum of tumor-bearing rats	0.63	23.9
Subcutaneous interstitial fluid	0.30	11.4
Tumor interstitial fluid	0.51	19.3

^a Four hours of incubation at 37° in formate buffer, pH 3.5.

^b Hyaluronic acid present in the initial solution, 1 mg/ml (2.64 μ moles/ml of *N*-acetylglucosamine).

determination of NAGA (5). In our assay, after 4 hr of incubation, the enzyme present in 1 ml of fluid was able to release from 12–40 % of the total NAGA present in the hyaluronic acid of the reaction mixture (Table I). Each determination was made in triplicate, and the mean values obtained are reported in Graph 1.

Results. The interstitial fluid sampled from the interscapular region before tumor trans-

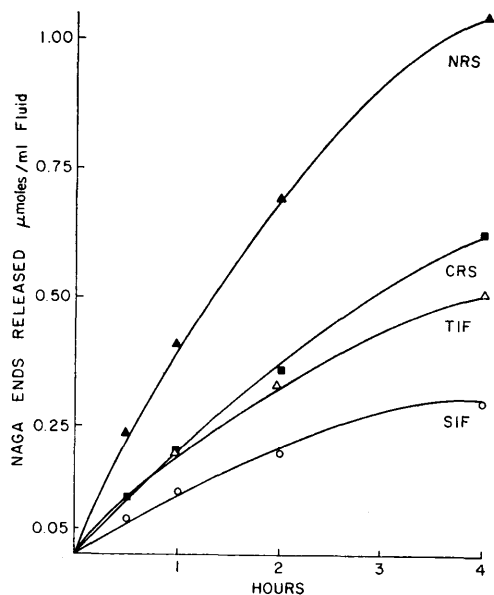


FIG. 1. Kinetics of the enzymatic hydrolysis of hyaluronic acid. The figure gives the micromoles per ml of *N*-acetylglucosamine (NAGA) liberated during the enzymatic action. NRS, normal rat serum. CRS, cancerous rat serum. TIF, tumoral interstitial fluid. SIF, subcutaneous interstitial fluid.

plantation released 0.07 ± 0.01 μ moles of NAGA per ml of fluid during the first 30 min of incubation. In the same period of time, the IF sampled from the same region after the tumor had incorporated the chamber, released 0.11 ± 0.01 μ moles of NAGA per ml. Under the same conditions of treatment, the serum of the tumor-bearing rat released also 0.11 ± 0.01 μ moles/ml of NAGA while the serum of the normal animal released 0.23 ± 0.01 μ moles/ml of NAGA.

Differences between normal and tumor-bearing animals were found for serum and IF at various intervals of incubation from 0.5 to 4 hr (Graph 1). The hyaluronidase activity of the serum in the normal animal was about four times the activity of the IF of the scapular region. In the tumor-bearing animals serum and IF from the tumor had practically the same hyaluronidase activity.

The increment of hyaluronidase activity produced by the development of the tumor was about 70 % as compared with the activity of the subcutaneous IF before transplantation. As expected (2, 6) the hyaluronidase activity of the serum in tumor-bearing animals was decreased by about 50 % as compared with the activity of the normal serum.

Discussion. The demonstration of hyaluronidase activity in Walker carcinoma and in the subcutaneous tissue depends on at least two conditions, the possibility of sampling the IF and the performance of the hyaluronidase assay in formate buffer at pH 3.5. The IF can be handled like blood serum, and the amount of *N*-acetylglucosamine released under our conditions of assay was 6–7 times higher than at pH 3.8. The incubation in acetate-phosphate buffer, pH 6.0, did not yield any depolymerization of hyaluronic acid. Hyaluronidase activity of the IF can be preserved for months if the sample is kept at -20° . The IF added to the incubation mixture at 4° still depolymerizes an amount of hyaluronic acid equal to about 25 % of the quantity depolymerized at 37° .

About 40 years ago Duran-Reynals proposed (7) that a "spreading factor" could be involved in the invasiveness of neoplastic cells (see also 8–10). Several investigators

(Review in 3) attempted to find hyaluronidase activity in tumors according to Duran-Reynals' hypothesis. The results were either negative, because the conditions of the assay were not optimal, or the data were uncertain because a bacterial hyaluronidase was present in the tumor (3). Our results showed that a high hyaluronidase activity was present in the IF of Walker carcinomas. In previous work (1) it was found that the hyaluronic acid content of the normal scapular interstitial fluid was about 50 $\mu\text{g}/\text{ml}$. Under the conditions of the experiments reported here, the hyaluronidase activity found in the same IF should be able to depolymerize in about 1 hr all the hyaluronic acid normally present in this fluid.

The reasons for the increase of the hyaluronidase activity in the IF of the tumor are not clear at this time. The possibility of a "leakage" of the enzyme from the lysosomes of the neoplastic cells has to be kept in mind since a similar mechanism was suggested for other lytic enzymes (11-13). The low level of hyaluronic acid previously found in the tumor IF may be related to the high hyaluronidase activity of this fluid.

A higher hyaluronidase activity in the tumor could also be responsible for an increase in small molecular weight fragments of hyaluronic acid released in the circulation. They could act as competitive inhibitors of serum hyaluronidase activity as previously

found in human (2) and confirmed in this work for rats.

Summary. The hyaluronidase activity of the interstitial fluid of Walker carcinoma transplanted into Sprague-Dawley rats was sharply increased when compared with the activity of normal subcutaneous interstitial fluid. On the contrary, the hyaluronidase activity in blood serum of tumor-bearing rats was depressed as compared with the activity of normal serum. The implications of these findings are briefly discussed.

1. Fiszer-Szafarz, B., and Gullino, P. M., in press.
2. Fiszer-Szafarz, B., Proc. Soc. Exp. Biol. Med. **129**, 300 (1968).
3. Cameron, E., "Hyaluronidase and Cancer," p. 65. Macmillan (Pergamon) (1966).
4. Gullino, P. M., Clark, S. H., and Grantham, F. H., Cancer Res. **24**, 780 (1964).
5. Reissig, J. L., Strominger, J. L., and Leloir, L., J. Biol. Chem. **217**, 959 (1955).
6. Herp, A., DeFilippi, J., and Fabianek, J., Biochim. Biophys. Acta **158**, 15 (1968).
7. Duran-Reynals, F., C. R. Soc. Biol. **99**, 6 (1928).
8. Lacassagne, A., Loiseleur, J., and Rudali, G., Bull. Cancer **44**, 552 (1957).
9. Vasiliev, J. M., Brit. J. Cancer **12**, 524 (1958).
10. Grossfield, H., J. Nat. Cancer Inst. **27**, 543 (1961).
11. Poole, A. R., and Williams, D. C., Nature **214**, 1342 (1967).
12. Sylvén, B., Eur. J. Cancer **4**, 463 (1968).
13. Bosmann, H. B., Exp. Cell Res. **54**, 217 (1969).

Received Sept. 12, 1969. P.S.E.B.M., 1970, Vol. 133.