Discussion and conclusions. In every instance the acute phase serum gave practically complete protection to all monkeys against a 20% monkey cord suspension which had usually been concentrated 10-fold by ultracentrifugation. A larger series of patients may show some without this titer of antibodies at, or before the onset of paralysis as did a group we reported who appeared to have been infected with a Lansing-like strain,[3] but among this group of 7 patients, ranging from 2 to 16 years of age, antibodies were definitely present either before infection or had already developed by the time of onset of paralysis.

The method of using serial 5-fold dilutions against a constant amount of virus, employed in the last 4 cases, appears to be satisfactory for this type of study and continued tests of this nature should afford important immunologic data on poliomyelitis. We realize that it would have been desirable to have known the titer of virus used in each instance, but we were influenced by consideration of the number of monkeys involved. It is obvious, however, from the results with serial serum dilutions, that the titer of virus used was neither in excess nor insufficient for the information sought.

Among the 4 cases in which serum titrations were made there is an apparent increase in antibody titer by the 45th, 74th and 80th days, but no definite rise in titer is apparent in the one case tested on the 24th day. In one instance the demonstrated increase in titer was only 5-fold, but in the others the increase was 25-fold or greater. It becomes apparent, therefore, that in poliomyelitis, as in most other virus infections, an increase in neutralizing antibodies usually develops as a result of infection. This finding encourages one to believe that a *type specific* serological test for this disease would have practical application. The frequent presence of specific antibody at readily detectable levels at the onset of paralysis or at the time of hospitalization lends further evidence against the possible usefulness of serum therapy, and possibly even serum prophylaxis. Obviously the results of these tests on man carry far more weight than tests on monkeys and chimpanzees following artificially induced infections.

[3] Hammon, W. McD., and Izumi, E. M., PROC. SOC. EXP. BIOL. AND MED., 1942, **49**, 424.

16683

Hyaluronidase Inhibitors in Body Fluids in Normal and Disease States.

JOHN K. FULTON, STANLEY MARCUS, AND WILLIAM D. ROBINSON.
(Introduced by W. J. Nungester.)

From the Department of Internal Medicine and the Rackham Arthritis Research Unit, Medical School, University of Michigan, Ann Arbor.*

Because of the probable relationship of hyaluronic acid to the formation and integrity of connective tissues, a relationship between hyaluronic acid metabolism and a large group of connective tissue diseases has been suspected, particularly in the case of rheumatic fever, rheumatoid arthritis, and disseminated vascular disease. The technical problems involved and the conflicting evidence which has been forthcoming have been extensively reviewed by Meyer.[1]

This report deals with attempts to relate this group of diseases to hyaluronidase activity, and, more significantly, with the apparent association of many malignant states in humans with disturbances of hyaluronidase inhibitors in the sera of such patients.

* The Rackham Arthritis Research Unit is supported by a grant from the Horace H. Rackham School of Graduate Studies of the University of Michigan.

[1] Meyer, K., *Physiol. Rev.*, 1947, **27**, 335.

Method. The method used depends on the observation of Seastone[2] that capsules of a group C streptococcus isolated from guinea pigs consist largely of hyaluronic acid and that hyaluronidases from a variety of sources exert a destructive effect on the capsule which is a function of time and enzyme concentration.

This principle has been utilized previously by McClean[3] for *in vivo* studies. In a discussion of methods for hyaluronidase estimation Meyer[1] has stated that the decapsulation method is unsatisfactory because of spontaneous decapsulation of the test organism.

The technic as now modified has proved to be free of this objection. It offers a highly sensitive method for quantitative assay of hyaluronidase concentrations in the range between .001 and .0001 viscosity reducing unit per cc. Since turbidity or viscosity of the test materials do not interfere, the method is particularly adaptable to comparative studies of hyaluronidase inhibitors in serum and other body fluids in normal and disease states.

The organism is maintained by daily transfer on rabbit blood agar plates. Only well isolated, large (1 mm diameter) mucoid colonies are fished for subculture to agar or broth. After overnight incubation, these surface colonies may reach as much as 3 mm diameter, and are soft, mucoid, and hemolytic. Blood agar cultures over 24 hours old are not suitable for transplanting to serum broth.

A heavy inoculum, consisting of from 5 to 10 large well isolated, mucoid colonies, is transferred to a tube of Difco Brain Heart Infusion broth enriched with 10% sterile heated (56°C/20 minutes) hog serum. The culture is incubated at 37°C for 4 to 6 hours. After incubation the culture tubes are centrifuged at high speed for 5 to 10 minutes, and the sediment resuspended in 2 to 3 cc of M/15 phosphate buffer, pH 6.5. This is the indicator suspension of encapsulated organisms. It is to be emphasized that in a properly prepared suspension every streptococcal chain is heavily encapsulated.

As Seastone found, capsules are better preserved during drying and fixing in the presence of blood or serum. This step can be eliminated by coating slides with a thin coat of Mayer's egg albumen before dropping loopfuls of bacterial suspension on them. The slides are then dried in air and stained with Wright's stain by the usual technic.

The enzyme used in these studies was a dried testicular hyaluronidase assaying 230 VRU/mg, supplied to us by Drs. VonderHeide and Rodney of the Parke-Davis Research Laboratories. A single batch has been used throughout these studies. However, other preparations from the Schering Laboratories gave comparable results.

To study enzyme inhibition 0.2 cc of enzyme containing 1.0 VRU/cc and 0.1 cc of the serum or its serial dilutions were added to oven-dried 13X90 mm tubes. Racks were shaken and then 0.1 cc of organism suspension was added to the tubes. Inhibition was expressed as the highest dilution of serum inhibiting the decapsulation of the test organism for 90 minutes.

Rheumatic Disease. Guerra[4] has reported that salicylates are capable of inhibiting the spreading action of hyaluronidase in skin. Using concentrations of buffered sodium salicylate from 5 to 100 mg%, no inhibition of hyaluronidase was demonstrable. (Table I) Inasmuch as Meyer[5] has described a gentisic acid conjugate of salicylate in urine capable of inhibiting hyaluronidase, the urine and serum of patients taking large doses of salicylates were tested for hyaluronidase inhibitors.

TABLE I.
Inhibition of Testicular Hyaluronidase *in vitro*.
Each tube: Enzyme 0.2 cc 1.0 VRU/cc
Organism susp. 0.1 cc
Drug 0.1 cc

	Conc. of drug mg %			
Sod. salicylate conc.	100	50	25	5
Inhibition obtained	0	0	0	0
Para amino benzoic acid conc.		50	25	5
Inhibition obtained		0	0	0
Gold sod. thiosulfate conc.	1.0	0.5	0.25	0.1
Inhibition obtained	0	0	0	0

[2] Seastone, C. V., *J. Exp. Med.*, 1939, **70**, 361.

[3] McClean, D., *J. Path. and Bact.*, 1942, **54**, 284.

[4] Guerra, F., *Science*, 1946, **103**, 686.

[5] Meyer, K., and Ragan, C., *Fed. Proc.*, 1948, **7**, 173.

TABLE II.
Inhibition of Testicular Hyaluronidase by Body Fluids Before and After Oral Sodium Salicylate.

	Dilution of body fluid			
	1:1	1:5	1:10	1:20
Normal serum	+	+	0	0
Normal serum after sod. sal.	+	+	0	0
Normal urine	0	0	0	0
Urine after sod. sal.	0	0	0	0

Time: 90 min.
Conc. of enzyme 1.0 VRU/cc.
+ = capsules present.

(Table II) In the case of urine no inhibitor capable of inhibiting 1.0 unit per cc of enzyme could be found, indicating that in therapeutically occurring concentrations salicylate conjugates presumably present in urine had no significant inhibitory effect on this reaction.

In serum, which normally contains hyaluronidase inhibitor, no increase could be produced by the ingestion of salicylates (Table II). These observations, therefore, confirm the observations of Pike[6] and Dorfman[7] and coworkers, who were unable to demonstrate hyaluronidase inhibition *in vitro* unless concentrations of salicylate of 2 g% were used.

Similarly, gold sodium thiosulfate and para aminobenzoic acid, drugs with an apparent influence on the course of certain connective tissue diseases, were without effect in producing enzyme inhibition when employed in therapeutically occurring blood serum concentrations. (Table I).

The presence of hyaluronidase inhibitor in normal serum has been described by several observers, but few quantitative observations have been made in disease states. Haas[8] described an inhibitor in the sera of normal and diseased individuals. However, there were no distinct differences between rheumatic fever and several other diseases listed. The inhibitor substance was about equally active against like amounts of pneumococcus type VI hyaluronidase and bovine testicular extract.

Friou and Wenner[9] were unable to find such inhibition of testicular hyaluronidase using the mucin clot prevention method, but found inhibition of streptococcal hyaluronidase in rheumatic fever, post-streptococcal convalescents as well as many normals. It is apparent from a perusal of this data that the inhibitor described was not the same as that which we have found, differing in the following characteristics: 1. The inhibitor described by Friou was not present in all sera, being absent from many normal sera. In our studies all sera have been inhibitory to some extent, although the variation among normals is marked. 2. The inhibitor was active against streptococcal hyaluronidase and inactive against hyaluronidase of testicular origin. In our studies the inhibitor was demonstrably active against testicular hyaluronidase as well as streptococcal enzyme. 3. The inhibitor was present in preserved sera as much as 10 years old and did not appear to decline in fresh sera on storage for 1 month. The inhibitor with which we deal is destroyed by heating to 56°C for 20 minutes and will disappear gradually in refrigerated sera within a few weeks. Inasmuch as the mucin clot prevention technic used by Friou requires relatively large amounts of substrate and enzyme, it is possible that the inhibitor present in all fresh sera toward testicular hyaluronidase was masked by its relative insignificance.

With these differences in mind, it is therefore proper to consider the testicular hyaluronidase - antihyaluronidase in a new relationship. As can be seen from the scatter diagram (Fig. 1) sera of various normal individuals of either sex vary widely in their ability to inhibit varying concentrations of testicular hyaluronidase. Since the same quantities of untreated serum, buffer, and bacterial substrate were present in each tube, these variations cannot be accounted for by variations in salt concentration. It is clear that any conclusions relative to the type of hyaluronidase inhibitors discussed here, in normal as compared to disease states must

[6] Pike, R. M., *Science*, 1947, **105**, 391.

[7] Dorfman, A., Reimers, E. J., and Ott, M. L., PROC. SOC. EXP. BIOL. AND MED., 1947, **64**, 357.

[8] Haas, E., *J. Biol. Chem.*, 1946, **63**, 163.

[9] Friou, G. J., and Wenner, H. A., *J. Infect. Dis.*, 1947, **80**, 185.

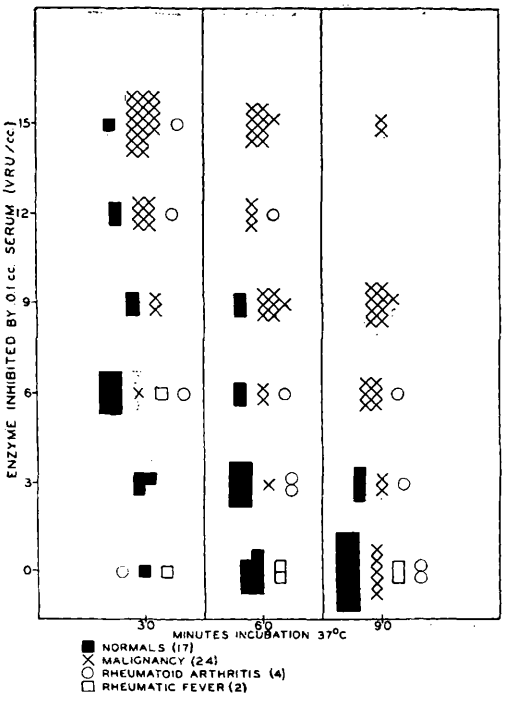

FIG. 1.
Relative testicular hyaluronidase inhibition by normal and disease sera.

take into account the wide range of normal variation.

When the sera of 4 active rheumatoid arthritis sera and 2 active and convalescent rheumatic fever sera were tested during the same test run with the group of 17 normals, they fell within the normal range. Because of the known instability of the inhibitor in stored sera, no attempt could be made to compare the sera of an active rheumatic fever patient with the same patient's convalescent serum weeks later. It seems evident that if changes do occur during the convalescence they are not outside the normal variation, which in itself remains unexplained. We, therefore, do not find evidence that either rheumatic fever or rheumatoid arthritis are typified by a clear cut disturbance in the particular hyaluronidase inhibitor with which this reaction is concerned.

Malignancy. Hyaluronic acid has been isolated from malignant tumors by Kabat[10] and by Meyer and Chaffee.[11] The presence of spreading factors (? hyaluronidases) in extracts of many animal and human tumors has been reported by Duran-Reynals.[12] The formation of spreading factor by malignant tissue has been regarded by Boyland and McClean[13] as the cause of the greater permeability of such tissue to nutrients.

On the basis of the unpublished observation of Rodney[14] that in Brown-Pearce carcinoma of rabbits a sharp rise in the titer of hyaluronidase inhibitor was detectable viscosimetrically in the sera of such animals, 24 sera of patients with a wide variety of malignancies both early and advanced and including leukemia and Hodgkin's disease were tested comparatively for hyaluronidase inhibitors with the same group of 17 control sera. As can be seen in the scatter diagram (Fig. 1), there is a statistical tendency for the cancer sera to show much greater inhibition under identical conditions. A few of the cancer sera show no characteristics which would distinguish them from normal sera, a few others are borderline, and some are markedly inhibitory. Since these observations have covered a relatively small group of normal, malignant, and other disease states, no conclusion can be drawn as to the exclusiveness of this property of excess inhibitor content for malignant sera, except to state that to date no other diseases studied have shown it.

Since testicular hyaluronidase was used throughout, to which no antibodies have been demonstrated, there is no evidence that an antigen-antibody reaction is involved, and the thermolabile nature of the inhibitor would suggest that it is not an antibody. The lack of differentiation between rheumatic states and normals would further suggest that the reaction is distinct from that described by Friou.

Conclusions. 1. A reliable biological method

[10] Kabat, E. A., *J. Biol. Chem.*, 1939, **130**, 143.

[11] Meyer, K., and Chaffee, E., PROC. SOC. EXP. BIOL. AND MED., 1939, **42**, 797.

[12] Duran-Reynals, F., and Stewart, F. W., *Am. J. Cancer*, 1931, **15**, 2790.

[13] Boyland, F., and McClean, D., *J. Path. and Bact.*, 1935, **41**, 553.

[14] Rodney, G., personal communication.

for the quantitative assay of small quantities of hyaluronidase or hyaluronidase inhibitor in the presence of body fluids is described.

2. Presumably, the conjugates of salicylates as well as salicylate itself in therapeutic concentrations have no inhibitory effect on testicular hyaluronidase *in vitro*.

3. Thermolabile hyaluronidase inhibitor has been found increased in a significant proportion of the sera of human malignancies in a small series studied.

4. Rheumatic states do not differ from the normal with respect to this inhibitor toward testicular hyaluronidase.

16684 P

Calcification of Hypertrophic Epiphyseal Cartilage *in vitro* Following Inactivation of Phosphatase and Other Enzymes.*

J. WALDMAN. (Introduced by F. C. McLean.)

From the Department of Physiology, The University of Chicago.

Mineralization of hypertrophic epiphyseal cartilage *in vitro*, first demonstrated by Shipley,[1] requires concentration of calcium ions and phosphate ions in excess of a critical ion product, assumed to approximate the solubility product of a calcium phosphate salt. The concept of calcification solely as a precipitation phenomenon, however, fails to account for the specific localization of the mineral.

Robison[2] demonstrated phosphatase in hypertrophic epiphyseal cartilage and achieved calcification by addition of phosphoric ester in minute amounts to media of subcritical ion product. Robison[3] regarded the local factor as possibly enzymatic since calcification *in vitro* had not been observed in tissues heated beyond 60°C, nor after complete inhibition of phosphatase by various chemical agents.

Gutman and co-workers[4] demonstrated phosphorylative glycogenolysis in hypertrophic epiphyseal cartilage and have contended that calcification *in vitro* utilizes this enzyme system. On the other hand, McLean *et al.*[5] have found little correlation of anaerobic glycolysis with calcification.

The present study was undertaken to determine whether or not enzymes are necessary for *in vitro* calcification.

Procedure. Slices of epiphyseal cartilage were taken from the long bones of young rats made rachitic on a modified diet 2965 of Steenbock and Black,[6] and were subjected to varied preliminary treatment. The slices were then incubated approximately 18 hours at 37°C in isotonic media containing known amounts of calcium and phosphate at pH 7.4. Following incubation the tissues were washed in distilled water and stained with 2% silver nitrate. Experiments were designed to compare calcification in inorganic phosphate with calcification in phosphoric ester (sodium glycerophosphate). In addition, extracts of epiphyseal cartilage were prepared and were

* This work has been supported in part by research grants from the Division of Research Grants and Fellowships of the National Institute of Health, U. S. Public Health Service; from the Josiah Macy, Jr. Foundation; and from the Williams-Waterman Fund of Research Corporation.

1 Shipley, P. G., *Bull. Johns Hopkins Hosp.*, 1924, **35**, 304.

2 Robison, R., *Biochem. J.*, 1923, **17**, 286.

3 Robison, R., and Rosenheim, A. H., *Biochem. J.*, 1934, **28**, 684.

4 Gutman, A. B., Warrick, F. B., and Gutman, E. B., *Science*, 1942, **95**, 461.

5 McLean, F. C., Lipton, M. A., Barron, E. S. G., and Bloom, W., unpublished data cited in The Biological Action of the Vitamins, University of Chicago Press; Chicago, 1942, p. 197.

6 Hess, A. F., Weinstock, M., Rivkin, H., and Gross, J., PROC. SOC. EXP. BIOL. AND MED., 1929, **27**, 140.