TABLE IV.
Apparent Free Histidine Plasma Values During ACTH Administration.
(10 patients with rheumatoid arthritis).

Patient	Total dosage, mg	Control values, γ per ml	Treatment values, γ per ml	Diff. between max. (control and treatment) γ per ml
H	310 (15)*	15.5 (15.3–15.8)	16.4 (11.5–18.1)	2.3
D	675 (17)*	16.4 (15.8–17.4)	16.2 (15.1–17.6)	0.2
G	640 (11)*	17.2 (15.7–18.7)	22.1 (19.4–23.9)	5.2
L	680 (11)*	19.0 (18.3–19.7)	18.5 (16.9–19.9)	0.2
P	600 (11)*	15.1 (15.0–15.2)	13.3 (12.3–14.5)	−0.7
K	620 (11)*	16.9 (16.7–17.0)	18.9 (18.3–19.9)	2.9
B	370 (12)*	19.7 (19.3–20.1)	16.3 (13.0–18.3)	−1.8
W	380 (12)*	12.1 (11.1–13.1)	13.1 (8.7–15.6)	2.5
F	370 (12)*	14.5 (14.3–14.7)	12.7 (10.3–14.9)	0.2
F	960 (11)*	24.5 (23.9–25.0)	27.4 (20.0–38.5)	13.5
Avg	560.5 (12.3)	17.1 (16.5–17.7)	17.5 (14.6–20.1)	2.5

* Days on treatment.

as yet been studied adequately.

Summary. 1. Five rheumatoid arthritics treated with Cortisone and 10 treated with ACTH show a striking increase in urinary excretion of apparent free histidine. This increase in histidine urinary excretion has to date occurred only in association with clinical remission.

2. Plasma levels were not significantly altered during the *average* treatment period, but the maximum value reached during treatment showed a significant increase when compared with the highest control values.

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Mucolytic Enzyme Systems XIII. Effect of Compounds on Hyaluronidase and its Inhibition by Human Serum.* (17877)

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During the course of the investigations on hyaluronidase and its inhibition, previously reported, further modifications of the procedures for the preparation of the substrate and the viscosimetric measurement of the hyaluronidase activity were instituted. These, as well as the effects of certain ions and or-

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ganic compounds on both the testicular enzyme activity and its non-antibody inhibitor in human blood serum, are now being reported. It has been established that salts exert a definite influence on hyaluronidase activity(1-7), and it is clear that the kind and degree of the influence depends largely on the salt concentration, the source and degree of purification of the enzyme and the substrate, and the method of determining the activity. These factors also affect the activity of the hyaluronidase inhibitor in blood serum(8-11). Haas(8) found that phosphate inhibits the hyaluronidase inhibiting property of serum, and Baumberger and Fried (9) showed that magnesium ions potentiate the serum inhibitor. Subsequently, the magnesium effect was studied more fully by Freeman *et al.*(11) who employed a turbidimetric method. In the present studies the viscosimetric method was used to observe the influence of phosphate and magnesium ions, different buffers, sulfhydryl radicals, cyanide, azide, histamine, benedryl, toluidine blue, and adrenocorticotrophic hormone (ACTH).

Methods. During warm weather, particularly, it had been found that batches of substrate solution suffered a spontaneous decrease in viscosity even though the solutions were stored in a refrigerator. The difficulty was traced to contamination with organisms that elaborate hyaluronidase. Accordingly, the procedure for making up the substrate solution was altered as follows: Approximately 2.5-3.0 g of hyaluronic acid powder prepared from human umbilical cords(12) was added to 250 ml of a buffer solution (6 vol. 0.1 M veronal buffer, pH 6.7, + 1 vol. 2.0 M NaCl) in a Waring blendor. After 1-2 min. agitation the mixture was poured out, 250 ml of buffer was used to rinse the blending vessel, and the rinsing liquid was added to the substrate solution in a flask which was then stoppered. This flask was heated at 65° for 30 min., allowed to stand at room temperature overnight, again heated at 65° for 30 min., and, while hot, passed first through a coarse porosity sintered glass filter, then through one of medium porosity. After allowing the filtrate to stand overnight, it is heated once more at 65° for 30 min. The substrate solution is now a little more viscous than desired, hence it is carefully diluted with the veronal-saline buffer until the "relative viscosity"(13) becomes 1.5. A veronal buffer is used with the substrate instead of the mixed phosphate-acetate buffers previously employed(13) since the veronal increased the sensitivity of both the enzyme and inhibitor measurements. The enzyme preparation was made as previously described(13) and the activity was adjusted (by either dilution with the buffer or strengthening with more bull testes powder) to give an R_0 value of 240 sec. R_0 is defined as the time required to reduce the "relative viscosity" of the reaction mixture to half its initial value. The final reaction mixture was prepared by adding 1.5 ml of the aqueous solution of the substance to be tested to 0.5 ml of the buffered enzyme, allowing to stand 6-10 min. at 38°, and then 4.0 ml of the buffered substrate was added. 5.0 ml of the mixture was pipetted into a viscosity tube for the measurements. The results were expressed as percent inhibition or activation of the hyaluronidase(12). For work on the serum inhibitor 0.04 ml of

1. Robertson, W. B., Ropes, M. W., and Bauer, W., *J. Biol. Chem.*, 1940, v133, 261.

2. Madinaveitia, J., and McClean, D., *Chem. and Ind.*, 1940, v59, 850.

3. Madinaveitia, J., and Quibell, T. H. H., *Biochem. J.*, 1941, v35, 453, 456.

4. McClean, D., and Hale, C. W., *Biochem. J.*, 1941, v35, 159.

5. Bergamini, L., *Arch. Scienze Biol.*, 1942, v28, 293.

6. Meyer, K., *Physiol. Rev.*, 1947, v27, 335.

7. Hadidian, Z., and Pirie, N. W., *Biochem. J.*, 1948, v42, 266.

8. Haas, E., *J. Biol. Chem.*, 1946, v163, 63.

9. Baumberger, J. P., and Fried, N., *J. Biol. Chem.*, 1948, v172, 347.

10. Dorfman, A., Ott, M. L., and Whitney, R., *J. Biol. Chem.*, 1948, v174, 621.

11. Freeman, M. E., Whitney, R., and Dorfman, A., PROC. SOC. EXP. BIOL. AND MED., 1949, v70, 524.

12. Wattenberg, L. W., and Glick, D., *J. Biol. Chem.*, 1949, v179, 1213.

13. Glick, D., and Gollan, F., *J. Inf. Dis.*, 1948, v83, 200.

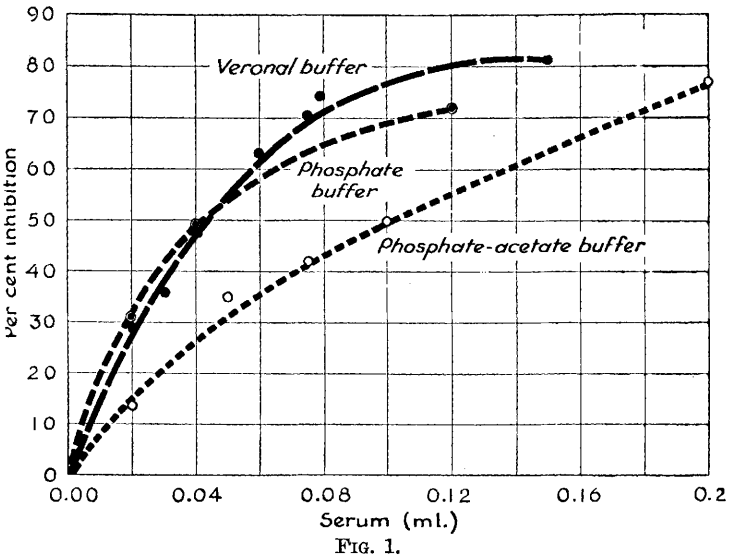

FIG. 1.

pooled normal human serum was diluted to 1.5 ml with distilled water. When the effect of a substance on the serum inhibitor was studied, the substance was first added to the serum solution before the latter was mixed with the enzyme. In those cases where the substance was acidic, the dissolved material was brought to pH 6.7 with 0.2 N NaOH before it was added to the serum solution.

Obtaining serum from a drop or two of blood for work on small animals or infants was carried out with the collaboration of Dr. M. L. Grais, as follows: Blood from a skin puncture was drawn up into glass tubing (10 cm long, 3 mm outer diam., 1.5 mm inner diam.), both ends of the tube were plugged with paraffin at once. After clotting, the tube was centrifuged, cut at the juncture of the packed cells and serum, and a serum aliquot was removed with a constriction pipette (14, p. 172). It was found in a number of cases that a sample obtained in this fashion from a heel prick in an infant showed no significant difference in the inhibitor concentration from a sample obtained by venipuncture of the same individual.

Results and discussion. Effect of buffer. A greater enzyme activity was observed when the phosphate-acetate buffer originally used (13) in the substrate solution (2 vol. 0.5 M phosphate, pH 7.0, 4 vol. 0.02 M acetate, pH 4.7, 1 vol. 2 M NaCl) was replaced by the veronal buffer described in this paper, or by a phosphate buffer (6 vol. 0.1 M phosphate, pH 6.7, 1 vol. 2 M NaCl), Fig. 1. At a pH of 6.7 veronal is at the acid end of its buffering range while phosphate is in the middle of its range. However, checks of the pH before and after the enzymatic hydrolysis of hyaluronic acid in solutions containing either buffer showed no change. Veronal was adopted in preference to the phosphate since the veronal was less favorable to the development of microorganisms.

Effect of magnesium and phosphate ions. The potentiating effect of low concentrations of magnesium ions on the serum inhibitor and the inhibiting effect of higher concentrations is illustrated in Fig. 2. The magnesium ion donated by the serum itself is negligible ($<1.5 \times 10^{-5}$ mEq./ml reaction mixture). Over the concentration range given in Fig. 2 it was found that magnesium ions had no direct influence on the activity of the hyaluronidase. An increasing direct inhibition of the enzyme was observed, however, with increasing concentrations of magnesium ion above 0.01 mEq. per ml reaction mixture. The influence of magnesium ions relative to

14. Glick, D., Techniques of Histo- and Cytochemistry, Interscience Publishers, Inc., New York, 1949.

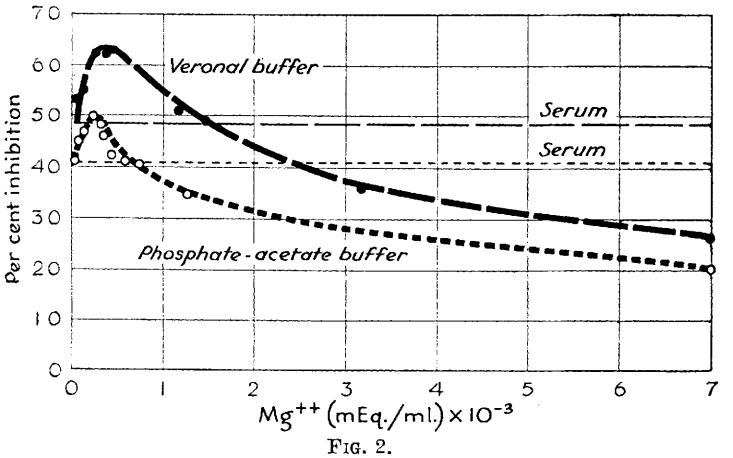

FIG. 2.

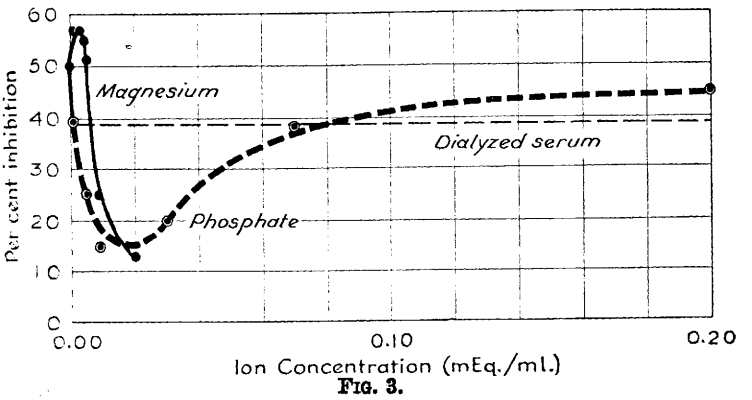

FIG. 3.

phosphate on serum dialyzed for 3 days at 6°, and chloride free, is illustrated in Fig. 3. From Fig. 4 it appears that the action of the serum inhibitor is reduced by a low concentration of phosphate, and then with increasing concentrations the inhibition is due solely to the effect of the ion on the hyaluronidase. The phosphate in the serum used would contribute about 2.5 x 10^{-5} mEq. per ml reaction mixture, and is therefore negligible.

Effect of sulfhydryl radicals. To determine whether sulfhydryl radicals are necessary for the hyaluronidase action, the effect of compounds that react with these groups was investigated. Chain and Duthie(15) had reported that 0.05 M iodoacetate inhibited the enzyme. It may be seen from Fig. 5 that iodoacetate destroys the serum inhibition while at the same time it inhibits the hyaluronidase. This would seem to implicate sulfhydryl in both the enzyme and inhibitor actions. The effect of other substances that react with sulfhydryl groups is given in Table I, No. 1-3. It is apparent that at the low concentrations used, these compounds have little direct effect on the hyaluronidase, and Nos. 1 and 3 exhibit a reduction of the serum inhibition. Whether No. 2 failed to affect the serum inhibitor because of the low concentration employed is not known. It was observed that in the presence of free cysteine or glutathione, hyaluronic acid spontaneously depolymerized. This is illustrated in Table II, and it serves to point up a disagreement with the results of Robertson *et al.*(16) who indicated that these substances are without

15. Chain, E., and Duthie, E. S., *Brit. J. Exp. Path.*, 1940, v21, 324.

16. Robertson, W. B., Ropes, M. W., and Bauer, W., *Biochem. J.*, 1941, v35, 903.

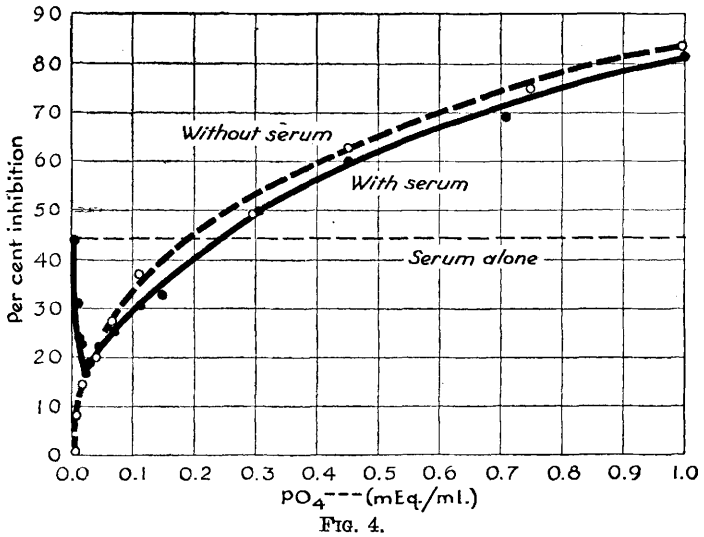

FIG. 4.

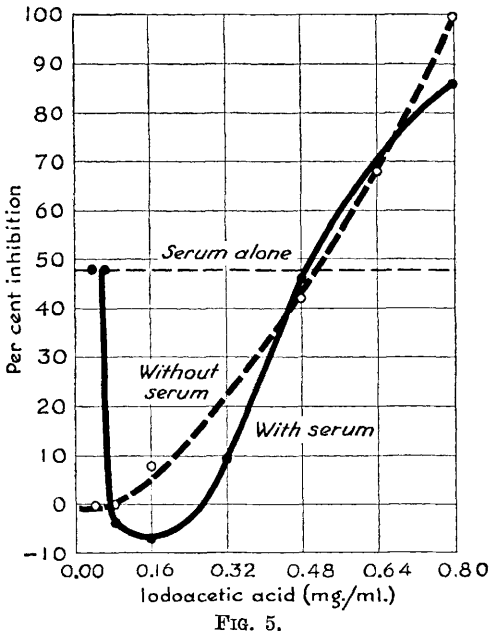

FIG. 5.

the depolymerizing effect; while it is not in disagreement with the work of Skanse and Sundblad(17) who found that the depolymerization by oxygen was enhanced by cysteine in the presence of traces of copper. The action of these reducing agents is similar to that of others(17), ascorbic acid and its oxidation products(3,15,16), copper(18,19), and azoproteins(20,21). Methionine, Table I, did not affect the viscosity of the substrate; at the concentration used, it did inhibit the enzyme and appeared to destroy most of the serum inhibitor.

Effect of other compounds. From Fig. 6 it may be seen that cyanide both destroys the serum inhibitor and inhibits the hyaluronidase. Chain and Duthie(15) had reported 0.05 M cyanide to be without effect on hyaluronidase, and Skanse and Sundblad(17) found that cyanide as well as diethyldithiocarbamate inhibited the depolymerization effected by oxygen, presumably by blocking heavy metal catalysis of this reaction. Sodium azide, Table I, also inhibits the enzyme in addition to inactivating the serum inhibitor, and the same applies to histamine and benedryl. Under the conditions they employed, Moynahan and Watson(22) found very little *in vitro* inhibition by benedryl and other antihistaminics on testicular hyaluronidase. Mayer and Kull(23) reported that, *in vivo*,

17. Skanse, B., and Sundblad, L., *Acta Physiol. Scand.*, 1943, v6, 37.

18. Romanini, M. G., *Arch. intern. pharmacodyn.*, 1949, v78, 427.

19. Pirie, A., *Brit. J. Exp. Path.*, 1942, v23, 277.

20. Claude, A., *J. Exp. Med.*, 1935, v62, 229.

21. Favilli, G., *Nature*, 1940, v145, 866.

22. Moynahan, E. J., and Watson, D., *Nature*, 1949, v163, 173.

23. Mayer, R. L., and Kull, F. C., PROC. SOC. EXP. BIOL. AND MED., 1947, v66, 392.

TABLE I.
Effect of Certain Compounds on the Hyaluronidase Activity and on the Serum Inhibitor.

No.	Compound	Amt per ml reaction mixture	% inhibition: Compound alone	% inhibition: Compound with serum	% inhibition: Serum alone
1	p-chloromercuribenzoic acid†	0.25 ml saturated soln.*	7	37	47
2	o-iodosobenzoic acid†	" " "	7	51	47
3	phenylmercuric nitrate	" " "	14	16	46
4	DL-methionine	12 mg	46	54	48
5	Sodium azide	2 "	31	39	53
6	Histamine dihydrochloride	2.5 "	17	26	46
7	Benedryl	2.5 "	17	17	46

* Saturated at 25°.
† Kindly supplied by Dr. L. Hellerman, Johns Hopkins University.

TABLE II.
Effect of Cysteine and Glutathione on the Viscosity of a Hyaluronic Acid Solution at 38°C.

Time from start of experiment	"Relative viscosity"*: Cysteine (5.1 mg cysteine HCl per ml reaction mixture)	"Relative viscosity"*: Glutathione (5.8 mg per ml reaction mixture)
0	1.50	1.50
5 min.	1.46	1.49
20 "	1.44	1.48
3 hr	1.14	1.33
11 "	0.79	0.94
18 "	0.56	0.69

* $\left(\frac{T_1-T_2}{T_1}\right)$ where T_1 equals initial outflow time from viscosity tube for complete reaction mixture, and T_2 equals outflow time for same mixture without substrate(12).

both pyribenzamine and antistine inhibited the spreading effect of hyaluronidase, while Swyer(24) observed that histamine increased the spreading action. It would appear that the physiological antagonism between histamine and the antihistaminic compounds is not reflected in a difference in their *in vitro* hyaluronidase inhibiting properties, even though their *in vivo* actions on the spreading phenomena may be in accord with this antagonism. Toluidine blue is of interest since it is a basic compound that might be able to compete with other basic factors in their reactions with hyaluronic acid. Fig. 7 demonstrates that toluidine blue can reduce the serum inhibition, and at the same time inactivate the enzyme. The data might be interpreted as indicating that the basic dye binds the acidic inhibitor and thus inactivates it, while the dye also competes with the enzyme for the acidic groups in the substrate and thus produces a direct inhibition of the enzyme action.

Effect of ACTH†. It has been found that ACTH and the adrenal cortex influence the level of the inhibitor in the serum(25). An *in vitro* test of the effect of ACTH on the

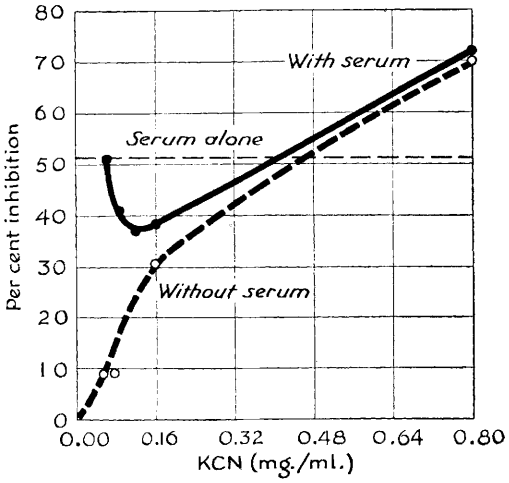

FIG. 6.

24. Swyer, G. I. M., *Biochem. J.*, 1948, v42, 28.

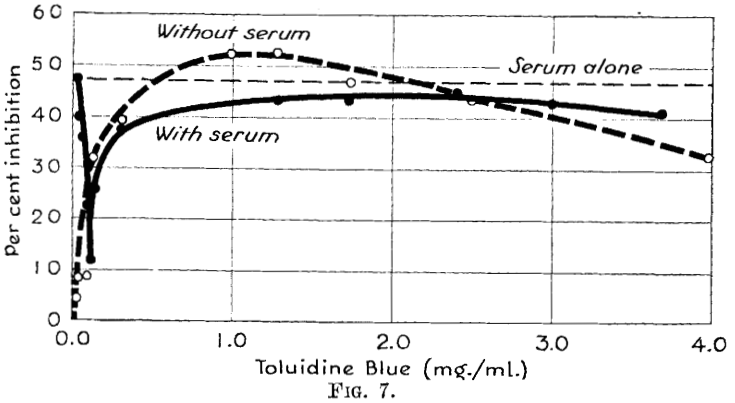

FIG. 7.

inhibitor as well as on the enzyme revealed a negligible influence on either up to the highest concentration used, 2.5 mg per ml reaction mixture. In an earlier investigation(12), cortisone and desoxycorticosterone were also found to be without effect. That adrenal compounds can influence the *in vivo* action of hyaluronidase as a "spreading factor" appears from the work of Opsahl(26) and Seifter *et al.*(27).

Summary. Modifications in the preparation of the substrate for the measurement of hyaluronidase activity by the viscosity method have been described. Increased sensitivity in the measurement of both the enzyme and its inhibitors has resulted, and spontaneous depolymerization of the substrate on storage has been virtually eliminated. The effect of the use of veronal and phosphate buffers in the substrate solution has been investigated, and the influences of a range of concentrations of magnesium and phosphate ions on both hyaluronidase and its serum inhibitor have been determined.

Evidence has been presented to indicate a possible role of sulfhydryl groups in both the enzyme and inhibitor actions. Cysteine and glutathione have been shown to cause a depolymerization of hyaluronic acid.

Methionine, cyanide, azide, histamine, benedryl, and toluidine blue have been found to both inactivate the serum inhibitor and inhibit hyaluronidase. Certain implications have been discussed.

Adrenocorticotrophic hormone has been shown to have negligible effect, *in vitro*, on either hyaluronidase or its serum inhibitor.

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25. Good, T. A., Good, R. A., Kelley, V. C., and Glick, D., *Fed. Proc.*, 1950, v9, 178.

26. Opsahl, J. C., *Yale J. Biol. Med.*, 1949, v21, 255, 433, 488; 1949, v22, 115.

27. Seifter, J., Baeder, D. H., and Begany, A. J., PROC. SOC. EXP. BIOL. AND MED., 1949, v72, 277.