a delayed reaction.

Summary. On incubating insulin under sterile conditions in acid saline with 2 moles of oxophenarsine (3-amino-4-hydroxyphenylarsenoxide hydrochloride) for each atom of sulfur contained in the insulin, it was found (by blood sugar determinations) that the effect of the treatment was to prolong the action on the blood sugar beyond that of untreated insulin. However, after 2 weeks standing this delayed action effect had disappeared and the material gave a blood sugar curve identical to that of the control insulin. Possible mechanisms of the interaction of oxophenarsine and insulin are briefly discussed.

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Failure to Relate Hyaluronic Acid to Elevated Erythrocyte Sedimentation Rate in Rheumatic Diseases.*

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It has been demonstrated that many asymmetric macromolecular substances are capable of increasing the erythrocyte sedimentation rate (E.S.R.) when added to normal blood. The substances listed by Fahraeus[1] as having such an action were gelatin, agar, gum arabic, gastric mucin, fibrinogen, globulin, and sodium caseinate. In recent years, pneumococcus capsular polysaccharides,[2] desoxyribonucleic acid,[3,4] and hyaluronic acid[4] have been shown to exert a similar effect. Meyer reported[4,5] that a testicular extract containing hyaluronidase, an enzyme complex which depolymerizes hyaluronic acid, when added to the blood of rheumatic fever patients decreased the elevated E.S.R. to normal limits. This report seemed of significance since hyaluronic acid is believed to be a constituent of the amorphous ground substance of the connective tissue, the site primarily involved in rheumatic fever.[6]

The investigation reported here was undertaken to determine whether hyaluronic acid is present in the blood of patients with rheumatic diseases and whether this substance is responsible for the elevated E.S.R. commonly observed.

Materials. The sodium salt of hyaluronic acid was prepared by a modification of the method of Seastone.[7] An aqueous extract of umbilical cord was deproteinized by stirring with a chloroform-amyl alcohol mixture in a Waring blender. The polysaccharide was finally precipitated by the addition of 2 volumes of ethyl alcohol saturated with sodium acetate in the cold. Sodium hyaluronate prepared in this manner produced a clear or opalescent, highly viscous solution at a concentration of 1.0% To avoid discrepancies due to variations in the preparation, one batch was made and used throughout the experiment.

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4 Meyer, K., Hahnel, E., and Feiner, R. R., PROC. SOC. EXP. BIOL. AND MED., 1945, **58**, 36.

5 Meyer, K., Chapter 18, *Currents in Biochemical Research*, ed. by Green, D. E., 1946.

6 Klinge, F., *Ergeb. d. allg. Path. u. Path. Anat.*, 1933, **27**, 1.

7 Seastone, C. V., *J. Exp. Med.*, 1939, **70**, 361.

The hyaluronidase§ was obtained from bull testes according to the method of Hahn.[8] The extract was dried while frozen and processed by the Madinaveitia method[9] with ammonium sulfate fractionation. The final enzyme preparation contained 233 turbidity reducing units per milligram.[10]

Methods. Erythrocyte Sedimentation Rate. E.S.R. was measured throughout these experiments by a modified Westergren technic,[11] with no correction for hematocrit.

Estimation of Hyaluronic Acid. Many acid mucopolysaccharides will co-precipitate with protein at an acid pH.[12,13] The resulting turbidity has been used by Kass and Seastone[10] as a quantitative measure of hyaluronic acid; a modification of their procedure was employed. Serial dilutions of sodium hyaluronate were made in normal rabbit plasma, 1.0 ml samples of which were then acidified by adding 9.0 ml of pH 3.1 acetate buffer (0.5 M). Concentration of the sodium hyaluronate in the several samples could be correlated with percent transmission read in a photoelectric nephelometer‖ with a 530 Å filter. In all cases, incubation of the serially diluted sodium hyaluronate with hyaluronidase (1.0 mg in 0.1 ml physiological salt solution) at 37° for one hour prior to acidification completely prevented the turbidity.

An attempt was made to use the carbazole reaction[14] to determine hyaluronic acid colorimetrically. By the direct method of Seibert and Atno,[15] using galactose as the standard, tests on serial dilutions of sodium hyaluronate in normal rabbit plasma showed that the procedure was not sensitive enough to measure accurately the small sodium hyaluronate concentrations needed to increase the E.S.R.

TABLE I.
Effect on Erythrocyte Sedimentation Rate of Adding Sodium Hyaluronate to Oxalated Rabbit Blood and Washed Erythrocyte Suspensions.

Sodium hyaluronate mg/ml	Erythrocyte sedimentation rate	
	Whole blood mm/hr	Washed erythrocytes* mm/hr
0.00	2	1
0.18	6	9
0.37	15	20
0.75	40	50
1.5	80	78
3.0	4†	0†

* Washed 3 times with physiological salt solution, then brought to volume in this diluent.
† Inhibition due to viscosity and clumping effects.

Preliminary Tests. To evaluate the reliability of the methods employed for the determination of hyaluronic acid in blood, experiments were carried out *in vitro* and *in vivo*. The increases obtained in E.S.R. on adding various dilutions of sodium hyaluronate to normal rabbit blood are shown in Table I. The apparent inhibition of the settling velocity in the highest concentration of the polysaccharide (3.0 mg/ml) was due to viscosity and clumping effects, since dilution with whole blood or physiological salt solution (P.S.S.) accelerated the E.S.R. Results similar to those in Table I were obtained with human blood. In every instance, following incubation with hyaluronidase, the settling velocity returned to normal. Hyaluronidase failed to affect the increased E.S.R. produced by gelatin or gum arabic.

In the *in vivo* studies, rabbits weighing 1 to 2 kg were injected intravenously with varying amounts of sodium hyaluronate. Increased E.S.R. and plasma turbidity could be demonstrated for varying periods after injection, as shown in Table II. In every case, the increased E.S.R. and the plasma turbidity were abolished by incubation with 1.0 mg of hyaluronidase at 37° for one hour.

Investigation of Blood from Clinical Cases. Specimens of oxalated blood were obtained

§ Kindly furnished for these experiments by courtesy of the Parke-Davis Company, Detroit, Mich.

8 Hahn, L., *Biochem. Z.*, 1943, **315**, 83.

9 Madinaveitia, J., *Biochem. J.*, 1941, **35**, 447.

10 Kass, E. H., and Seastone, C. V., *J. Exp. Med.*, 1944, **79**, 319.

11 Westergren, A., *Acta Med. Scandinav.*, 1920, **54**, 247.

12 Meyer, K., and Palmer, J. W., *J. Biol. Chem.*, 936, **114**, 689.

13 Meyer, K., Palmer, J. W., and Smyth, E. M., *J. Biol. Chem.*, 1937, **119**, 501.

‖ Lumetron, Model 400A, Photovolt Corp., New York City.

14 Dische, Z., *Mikrochemie*, 1930, **8**, 4.

15 Seibert, F. B., and Atno, J., *J. Biol. Chem.*, 946, **163**, 511.

TABLE II.
Intravenous Injection of Sodium Hyaluronate in Rabbits. Inhibition of Increased Erythrocyte Sedimentation Rate and Plasma Turbidity by Hyaluronidase.

Rabbit	Sodium hyaluronate injected, mg/kg	Time after injection	Erythrocyte sedimentation rate after incubation with		Plasma turbidity after incubation with	
			P.S.S.,* mm/hr	Hyaluronidase,* mm/hr	P.S.S. % transmission	Hyaluronidase, % transmission
1	72.2	40 min.	72	1	54	100
		6 hr	15	5	69	100
		96 "	1	—	100	100
2	72.4	3 min	42	1	42	100
		1 hr	53	1	47	100
		6 "	15	1	62	100
3	38.7	30 min	12	1	61	100

* See text.

from 16 cases of rheumatic fever,¶ 16 cases of rheumatoid arthritis, and 10 patients with various other diseases. The initial E.S.R. was first determined. To investigate the effect of hyaluronidase, 1.0 mg in 0.1 ml P.S.S. was added to 1.0 ml of the blood and as a control, 0.1 ml P.S.S. alone was added to an equal volume of blood. These specimens were incubated at 37°C for one hour and the E.S.R. again measured. As indicated in Table III, in most cases where there was a significant alteration of the E.S.R. by hyaluronidase, the P.S.S. alone produced a similar effect.

The turbidimetric method described earlier for estimating hyaluronic acid in plasma was applied to two 1.0 ml samples of plasma from the same blood. To one was added hyaluronidase; to the other P.S.S., as above. After incubation at 37°C for one hour, the plasmas were acidified with the acetate buffer. In no case did the two specimens show a significant difference in the turbidity formed. Rarely was there a measurable difference between the results obtained with the pathologic specimens and those from normal individuals.

It has been reported[16,17] that many blood specimens contain antinvasin, a substance which inhibits hyaluronidase activity. Hyaluronidase inhibition by human or rabbit plasma was not encountered under the experimental conditions in this study.

Many patients involved in this study had been on salicylate therapy. Since this therapeutic agent has been reported to have a hyaluronidase-inhibiting activity,[18,19,20] experiments were carried out to determine whether it had been responsible for the failure of hyaluronidase to alter the E.S.R.

Varying quantities of sodium salicylate (10% in P.S.S.) and sodium hyaluronate were injected intravenously into rabbits, blood was drawn by cardiac puncture at intervals, and salicylate determined by the method of Brodie *et al.*[21] Although salicylate affected the elevated E.S.R. produced by sodium hyaluronate, it did not alter hyaluronidase inhibition of E.S.R. and plasma turbidity (Table IV). This is in agreement with the results of Pike[22] and would seem to rule out salicylate inhibition as a factor in the failure of hyaluronidase to alter significantly the E.S.R. of the clinical blood specimens tested. These findings do not rule out the possibility of interference with the oligosaccharase action of the hyaluronidase

¶ Obtained through the courtesy of Dr. P. V. Wooley of Children's Hospital, Detroit, Mich.

16 Haas, E., *J. Biol. Chem.*, 1946, **163**, 63.

17 Haas, E., *J. Biol. Chem.*, 1946, **163**, 101.

18 Guerra, F., *Science*, 1946, **103**, 686.

19 Guerra, F., *J. Pharm. Exp. Therap.*, 1946, **87**, 143.

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

21 Brodie, B. B., Udenfriend, S., and Coburn, A. F., *J. Pharm. Exp. Therap.*, 1944, **80**, 114.

22 Pike, R. M., *Science*, 1947, **105**, 391.

TABLE III.
Effect of Hyaluronidase on Elevated Erythrocyte Sedimentation Rate of Blood from Patients with Various Diseases.

Disease condition	Patient	Erythrocyte sedimentation rate		
			After incubation with	
		Initial, mm/hr	Hyaluronidase,* mm/hr	P.S.S.,* mm/hr
Rheumatic fever	1	68	90	83
	2	113	122	115
	3	42	46	37
	4	95	71	90
	5	29	22	14
	6	18	8	7
	7	8	3	2
	8	19	8	8
	9	18	18	30
	10	74	67	55
	11	44	47	—
	12	45	24	16
	13	34	34	25
	14	26	8	10
	15	16	3	3
	16	17	9	5
Rheumatoid arthritis	17	52	34	34
	18	76	79	74
	19	106	122	112
	20	88	62	53
	21	40	40	36
	22	20	12	12
	23	20	15	2
	24	92	86	75
	25	38	42	37
	26	—	35	32
	27	38	33	32
	28	65	52	48
	29	85	80	63
	30	—	72	69
	31	105	107	105
	32	28	29	30
Acute disseminated lupus erythematosus	33	67	55	70
Upper respiratory infection	34	44	41	37
Pulm. tuberculosis	35	30	42	34
	36	62	88	79
Hodgkins disease	37	140	135	127
	38	95	110	95
Lymphosarcoma	39	52	27	27
Aplastic anemia	40	130	140	130
Pernicious anemia	41	41	29	34
	42	59	57	59

* See text.

complex.[23] Salicylates have been reported to decrease the E.S.R. of pathologic blood specimens.[24] The explanation of this phenomenon is obscure and is under further investigation.

[23] Hahn, L., *Arkiv. Kemi. Mineral. Geol.*, 1946, **22**A, No. 1; *Ibid.*, 1946, **22**A, No. 2.

[24] Homburger, F., *Am. J. Med. Sc.*, 1945, **210**, 168.

TABLE IV.
Failure of Sodium Salicylate to Alter Action of Hyaluronidase on Increased Erythrocyte Sedimentation Rate and Plasma Turbidity Following Intravenous Injection of Sodium Hyaluronate.

Rabbit	Sodium hyaluronate, mg/kg	Time after injection	Salicylate level, mg %	Erythrocyte sedimentation rate after incubation with		Plasma turbidity after incubation with	
				PS.S.,* mm/hr	Hyaluronidase,* mm/hr	P.S.S., % transmission	Hyaluronidase, % transmission
1	91.7	30 min.	37.5	1	1	37	100
		5 hr	16.7	2	2	90	100
2	66.6	1 "	5.6	38	1	48	100
		2 "	4.6	10	1	66	100
3	62.5	1 "	52.8	15	2	52	100
		5 "	33.4	12	2	66	100
4	82.3	1 "	73.8	1	1	53	100
		5 "	43.0	12	1	63	100

* See text.

Discussion. The data presented in this report fail to substantiate the possibility that hyaluronic acid, at least in a polymerized form, is present in the blood of patients with rheumatic fever, rheumatoid arthritis, and several other diseases (Table III) as has been suggested by Meyer.[4,5] The polysaccharide could not be demonstrated in the blood of normal individuals.[25] Depolymerized hyaluronic acid would not be demonstrated by the methods employed here, but previous work[1] and our own indicate that the depolymerized acid will not increase E.S.R. to a marked degree. In a personal communication, Dr. Karl Meyer has stated that in studies with his preparations of hyaluronidase, spreading activity and E.S.R. inhibition did not run parallel. He was able to separate the factor that decreased E.S.R. from hyaluronidase activity by adsorption on lead sulfide.

Summary. 1. Hyaluronic acid is apparently not responsible for the increased erythrocyte sedimentation rate of blood from patients with rheumatic fever and rheumatoid arthritis. 2. Sodium salicylate failed to inhibit the effect of hyaluronidase on elevated E.S.R. produced in rabbits by the intravenous injection of sodium hyaluronate.

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Immunologic Reactions Following the Intradermal Inoculation of Influenza A and B Vaccine.*

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It has been shown[1] that the intradermal injection of heat-inactivated mumps virus into human beings may exert a pronounced antigenic effect. Data are[2] available which sug-

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2 Stokes, J., and Enders, J. F., 1947, unpublished data.