TABLE III.
Effect of Glutamine, Asparagine, Arginine, and Proline on Anaerobic Growth of *E. coli* in the Absence of CO_2.

Compound	Glutamine	Asparagine	Arginine	Proline	Control
Growth	125	40	55	30	5

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter. Compounds were added in 2-mM quantities.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

TABLE IV.
Replacement of CO_2 by Citric Acid Under Aerobic and Anaerobic Conditions.

Organism	Citric Acid	
	Nitrogen with O_2 and CO_2 removed	Air with CO_2 removed
Escherichia coli	9	4
Aerobacter aerogenes	180	50

The extent of growth is indicated by turbidimetric readings on the photoelectric colorimeter. 2-mM quantities of citric acid were used.
Total volume, 25.5 cc. Incubation time, 12 hr. Temp., 37°C.

fact that oxalacetic by-passes the CO_2 requirement to such an extent indicates that this acid may function as the chief substrate for protein synthesis under anaerobic conditions.

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Magnesium and Hyaluronidase Inhibitor of Blood Serum.

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Numerous reports[1-12] have established the presence of a hyaluronidase inhibitor in the blood sera of several species. The nature of the inhibitor has not been elucidated: but evidence has been presented that it is not a specific antibody;[2,13-16] nor an enzyme specifically attacking hyaluronidase.[6,9,12] The

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TABLE I.
Hyaluronidase Inhibitor Activity of Human Blood Serum Fractions in the Presence of Different Amounts of Magnesium Ion.

	No added magnesium		Additional magnesium	
	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum
Serum A	4.4	70	68	150
Component IA	.8	7	60	28
" IIA	.8	5	60	75
" IA and IIA	.8	10	60	100
Residue	2.9	—	—	—

inhibitor may be a polysaccharide or protein-polysaccharide complex successfully competing with the hyaluronic acid as an alternate substrate.[3,7,12]

Partial separation of serum into two fractions that were inactive alone, but active when recombined, seemed to indicate that the inhibition was probably more complicated than originally supposed. Both fractions were first reported to be of protein nature:[9] Component I was flocculated from serum at −2°C, pH 8.4, and 25% ethanol; followed by component II flocculated by lowering the pH to 6.1. Another report[10] showed that citrated, oxalated, and dialyzed serum was not active unless magnesium was added. Studies in these laboratories indicated that when human blood serum was fractionated according to method of Cohn *et al.*,[17] much of the activity appeared in fractions II and III; but only if the fraction was dissolved in a solution containing magnesium ions.

Further experiments on the fractionation of normal human serum with respect to the hyaluronidase inhibitor and the contribution of magnesium to the activity of the inhibitor are herewith reported.

Methods. Twenty frozen stored samples of human blood sera were selected at random from a series originally obtained from young adult males. The hyaluronidase, from frozen bovine testis, was prepared, assayed, and standardized by previously described methods.[18,19] Sera were assayed for hyaluronidase inhibitor by incubating suitable dilutions with standardized hyaluronidase, subsequently assaying the reaction mixture for active hyaluronidase.[12] Dilutions were made with saline-borate buffers with and without measured amounts of magnesium chloride. Magnesium content of the undiluted serum and its fractions was determined with titan yellow.[20] Thus, the relative amounts of serum and magnesium present in the reaction mixture could be calculated and the relation expressed as millimoles of magnesium per ml of reaction mixture. The inhibitor activity was calculated as units of hyaluronidase inactivated per ml of serum.[19] Increased amounts of magnesium were added to diluted samples of serum until 4 or 5 successively higher magnesium concentrations gave no further increase in inhibitor activity.

Normal human serum was buffered to pH 8.4 with barbiturate, cooled to 0°C, and component I was flocculated by the slow addition of an equal volume of 50% ethanol. After separating and washing the precipitate in a refrigerated centrifuge, the supernate was brought to pH 6.1 with 0.6 *M* acetate buffer, pH 4.0. The precipitate that formed, component II, was removed and washed in refrigerated centrifuge. The two precipitates were suspended in volumes of 0.15 *M* saline equal to the volume of the original serum and

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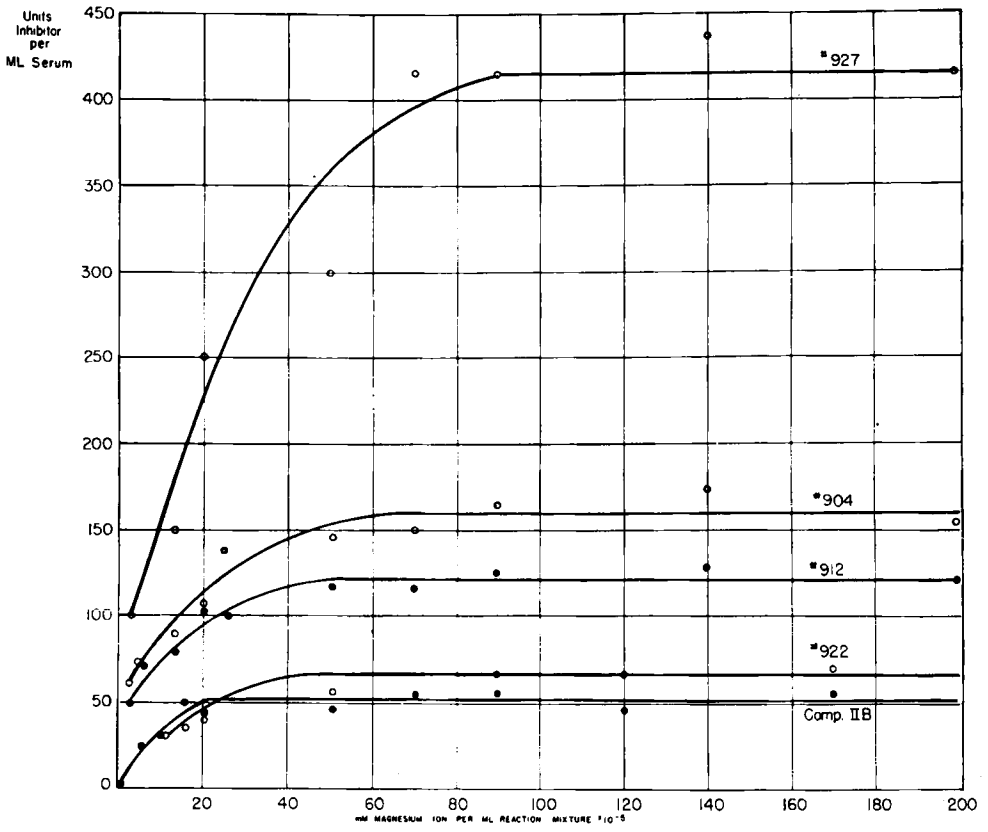

FIG. 1.
Relation of magnesium concentration to inhibitor activity in assay reaction mixture.

assayed for hyaluronidase inhibitor with and without additional magnesium. Serum components and supernate were analyzed for magnesium and assayed for inhibitor with the results shown in Table I as units of inhibitor per ml of serum and millimoles of magnesium per ml of the reaction mixture.

Table I illustrates the values found in 3 fractionations of normal human sera. The original sera contained approximately 80.10^{-5} millimoles of magnesium ion per ml. This is 18 times the magnesium ion content of the assay reaction mixture after suitable dilution of the serum with saline. This reaction mixture assayed 70 inhibitor units per ml of serum. In order to obtain the maximum inhibitor activity of 150 units per ml of serum, it was necessary to increase the magnesium concentration of the assay mixture to at least $60 + 10^{-5}$ millimoles per ml by diluting the serum with saline-magnesium solutions instead of saline alone.

The washed precipitates of component I contained small amounts of magnesium; less than one-fifth of the total serum magnesium. It was apparent that the precipitates contained insufficient magnesium when suitably diluted with saline for assay. When these precipitates were diluted with saline containing sufficient magnesium to support maximum inhibition, only a part of the total inhibitor was found.

Component II precipitates also contained minimum amounts of magnesium and when diluted with saline alone gave little indication of inhibitor activity. When diluted with saline-magnesium solution, however, these precipitates gave inhibitor values equal to at least half of the total activity of the serum. When components I and II were mixed, the

TABLE II.
Hyaluronidase Inhibitor Activity of Blood Sera With and Without Added Magnesium.

	Normal blood serum		Added magnesium	
Sample	mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum	Total mM of Mg ion per ml reaction mixture, $\times 10^{-5}$	Hyaluronidase inhibitor units per ml serum
925	5.0	43	65	75
920	3.5	24	66	87
917	4.1	40	68	110
929	4.1	50	67	112
918	4.1	50	68	116
916	5.2	47	68	117
934	4.1	73	67	145
919	3.5	71	68	148
921	3.3	63	68	150
932	4.1	78	67	150
923	4.2	62	68	165
926	4.5	63	68	167
924	5.0	75	68	175

saline dilutions were again very low in magnesium; and consequently, showed little activity. When sufficient magnesium was added, the activities of the mixtures were greatly enhanced.

These experiments clearly indicated that the hyaluronidase inhibitor in human serum and in the active serum fractions required some minimum concentration of magnesium ion for expression of its maximum activity *in vitro*. The actual amounts of magnesium required for maximum activity were determined by experiments illustrated in Fig. 1. These data demonstrated that when sera, or its active fractions, were diluted with saline alone, very low inhibitor activities were found in the assay reaction mixtures. Increasing amounts of magnesium in the reaction mixture were accompanied by regular increments of activity until a maximum activity was reached where the reaction mixture contained about 50 to 60 x 10^{-5} millimoles of magnesium ion per ml. Further increases or decreases in activity were within the experimental error of the assay up to concentrations of 200 x 10^{-5} millimoles.

It should be noted, however, that the assay method employed demanded an inhibitor concentration of only 2 to 4 units per ml in reaction mixture. This restriction necessitated a 20 to 100-fold dilution of most sera. Thus, when the serum was diluted with saline alone, the magnesium concentration, about 80 x 10^{-5} millimoles per ml of serum, fell to less than 5 x 10^{-5} millimoles per ml of the reaction mixture. Consequently, low inhibitor values resulted. Maximum inhibitor values were found when the magnesium concentration was increased to 60 x 10^{-5} millimoles per ml, very near that of the original serum. This was clearly shown in Fig. 1 where data were plotted for component IIB and 4 sera of high, low, and intermediate activity.

Thirteen additional samples were determined by diluting the sera with saline alone, and by diluting with saline containing adequate concentrations of magnesium. These data, showing the necessity of magnesium in the inhibitor assay, are given in Table II.

Summary. Magnesium ion has been found to be essential for the hyaluronidase inhibitor activity of human blood under the conditions of the *in vitro* assay. The minimum amount of magnesium required for expression of maximum activity has been determined for the assay method employed in these laboratories.