quots of the first butanol extract of the shock plasma obtained from one of the dogs were chromatographed with a standard series of histamine samples. A butanol sample containing 7.8 μg histamine (calculated on the basis of bioassay of the plasma) showed a chromatogram falling between standard chromatograms of 5 and 10 μg histamine and corresponding most closely to the 7 μg standard as judged by an unbiased observer. The chromatographic method with a limit of sensitivity of 2-3 μg per sample is obviously inferior to bioassay in this respect but is superior so far as specificity is concerned. The demonstration of histamine release in amounts roughly equivalent to those observed in the anaphylactic experiments in a high percentage of the trypsin shock experiments confirms an earlier report from this laboratory(3) but is in contrast to a more recent report, also from this laboratory.(11) We are not at present prepared to explain the discrepancy.

Summary. A method for the chemical identification of histamine in blood by paper chromatography is described in detail and experiments are presented to show the release of relatively large amounts of histamine in dogs undergoing anaphylactic and trypsin shock. Peptone, when injected into dogs in amounts sufficient to produce severe shock, interfered with the identification of histamine which could, however, be found on the chromatograms after addition of small amounts of peptone to rabbit blood (*in vitro*).

The author wishes to express his appreciation to Dr. Carl A. Dragstedt for many helpful suggestions.

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Inhibition of Hyaluronidase by Aromatic Compounds. (17521)

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The aim of our experiments was to find substances inhibiting the action of hyaluronidase. Repeating Guerra's work,(1) we(2) confirmed his observations that this enzyme was inhibited by sodium salicylate. Prior to this, hyaluronidase was found to be inhibited by heparin, gastric mucin, pseudo-globulin,(3) morphine,(4) hyaluronic acid,(5) estrogens(6) and, of course, enzyme inactivators. These experiments were carried out *in vivo*. By studying the effect on the area of spread in the skin, it was found that ascorbic acid,(3) glycerin,(7) triacetin,(7) arsenious oxide,(7) lecithin,(8) peptones,(8) urethane, sodium diazobenzene sulfonate,(9) hirudin,(7) human urine(10) and diazotized proteins(11) enhance this enzymatic action. Obviously, these lists fail to reveal chemical structures which are essential for anti-hyaluronidase activity. We, therefore, undertook to study a series of simple chemically related compounds and used the turbidimetric method described by Kass and Seastone.(12)

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It is based on the observation that pure hyaluronate in a buffered solution produces a fairly stable colloidal suspension when mixed with diluted acidified horse serum. There is a reduction in turbidity when hyaluronate is depolymerized by hyaluronidase. The efficiency of various inhibitors can be studied by measuring the turbidity of the various mixtures when compared to controls. This turbidity is a function of the undecomposed hyaluronic acid present. When the enzyme is introduced into the system, the turbidity produced holds an inverse relationship to the hyaluronidase content.

To determine the effect of a particular chemical, a series of freshly prepared dilutions of the enzyme were prepared. Five tubes were used with serial dilutions running in the order of 10, 20, 30, 40 and 50 γ of enzyme per cc. Since the concentration of the original solution was 100 γ per cc, 0.1, 0.2, etc. of this solution was placed in the tube and diluted to 0.5 cc with buffer pH 6.0 to maintain constant volumes. To each of these dilutions, 0.5 cc of the hyaluronate solution (0.04 mg/cc) was then added. Immediately after this, 0.1 cc of the compound to be tested was added to another tube which contained 50 γ of hyaluronidase and the substrate. All tubes were then incubated for 30 minutes in a constant temperature water bath at 37.5°C. The remainder of the procedure was performed as outlined by Kass and Seastone. Whenever possible, several determinations were made the same day. Each day a curve was plotted anew. This was desirable because of the variability and colloidal instability of proteins in the diluted horse serum which served as an indicator for undecomposed hyaluronic acid present. All the curves were S shaped in form and were prepared by using the dilution as abscissa and the turbidity, in terms of the reading on the Klett-Summerson photo-electric colorimeter, as ordinate. Red filter No. 66 was used.

As an example, the curve obtained using sodium salicylate is illustrated in Fig. 1. Here the reading on the colorimeter corresponding to 50 γ of hyaluronidase is 35. When 10 mg of sodium salicylate was added, the reading was 47, which corresponds to a

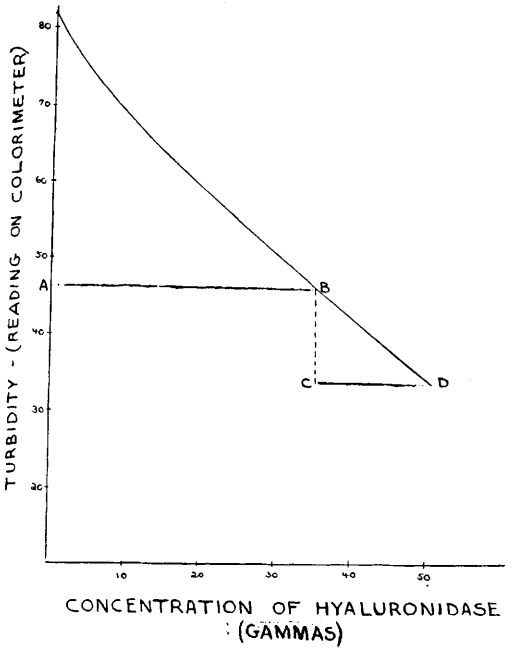

FIG. 1.

Method used to calculate activity of inhibitors.
D—Turbidity equivalent to 50 γ of hyaluronidase.
B—Turbidity obtained by the addition of sodium salicylate.
AB—Quantity of hyaluronidase remaining after using sodium salicylate.
CD—Quantity of hyaluronidase inactivated by 10 γ of sodium salicylate.

total quantity of 34.7 γ of enzyme. This difference must have been inactivated by the sodium salicylate added. From the concentration and volume of the inhibitors, the amount of enzyme inactivated by 1 mg can be calculated. This procedure was repeated for each one of the various inhibitors tested.

Before any inhibitor was used, certain criteria were established to eliminate invalidating factors. These were as follows:

1. Solutions of the inhibitor must be clear.

2. Alcohol solutions were diluted 1:10 with buffer pH 6.0 to ascertain whether mere dilution produced a precipitate.

3. Non-specific protein precipitating action of the inhibitors were tested by adding them to the acidified normal horse serum which was diluted with 3 parts of buffer pH 4.2.

4. Extensive studies of all pHs were carried out (Table I). Two buffers of pH 6.0 and 4.2 were checked periodically on a Beckman

TABLE I.
Comparative List of Hyaluronidase Inhibitors.

%	Inhibitor		pH of buffer (pH 6.0) + inhibitor	pH of mixture: buffer pH 6.0 + buffer pH 4.2 + inhibitor	γ of hyaluronidase inactivated by 1 mg of inhibitor
1	m-hydroxybenzoic acid	alc	5.52	4.25	3.2
1	p-hydroxybenzoic acid	"	5.65	4.25	3.6
1	ethyl p-hydroxybenzoate	"	6.12	4.26	7.0
5	phenol	aq	6.02	4.23	7.5
5	resorcinol	alc	6.12	4.25	8.4
1	o-nitrophenol	"	6.06	4.25	19.7
1	guaiacol	aq	6.02	4.24	24.0
1	m-cresol	"	6.03	4.26	25.0
1	methyl p-hydroxybenzoate	alc	6.12	4.25	28.0
1	propyl p-hydroxybenzoate	"	6.12	4.25	31.0
1	4-aminosalicylic acid	"	5.68	4.26	31.7
1	o-cresol	"	6.12	4.26	37.5
1	dinitrophenol	"	5.72	4.25	39.6
1	p-chlorophenol	"	6.12	4.25	42.0
1	o-chlorophenol	"	6.13	4.25	44.2
1	picric acid	aq	5.68	4.26	46.4
1	butyl p-hydroxybenzoate	alc	6.12	4.25	93.0
0.1	hexylresorcinol	"	6.12	4.26	400.0
.025	diamyl phenol	"	6.12	4.27	600.0
.052	2,5 ditertiary butylhydroquinone	"	6.10	4.27	669.3
10	sodium salicylate	aq	6.02	4.26	1.5
5	sodium gentisate	"	5.85	4.27	0.7

pH meter. The readings were between 5.95 and 6.02 for the former and 4.15 and 4.20 for the latter.

To ascertain the pH of the solution containing buffer pH 6.0 and the same proportion of inhibitor as in the studies, *i.e.* 1:0.1, a thorough investigation was undertaken with each inhibitor (Table I) and the values obtained varied between 5.52 and 6.13. It is important also to note that there is no direct relationship between the variations in pH and the potency of drug inhibition and that the activity of hyaluronidase is maximal within these two extremes.

When the two buffers (6.0 and 4.2) were mixed in the same proportion as in the determinations, *i.e.* 1:3, the average pH value of the mixture was 4.25 ± 0.02. The effect of each inhibitor on this value was then studied by adding 1:10 of the inhibitor to the mixed buffers. As can be observed, the values are between 4.22 and 4.27 and well within the limits of experimental error under the conditions of this experiment. Thus, the influence of the inhibitor to displace the pH of the buffering system was eliminated and the action on the acidified horse serum was a variable of the enzyme factor alone; nonspecific action of the inhibitors was eliminated.

All data are tabulated in terms of gammas of hyaluronidase inactivated by one milligram of each compound. Since ethyl alcohol (95%), in the volume used, had no effect on the production of turbidity, it was used as a solvent for most of the drugs. In all other cases aqueous solutions were used.

Discussion. At this time we may speculate on the clinical importance of these findings. If an etiological relationship can be presupposed between hyaluronidase and acute rheumatic fever, the need for massive doses of sodium salicylate should be explained by our findings, since this drug is a weak inhibitor of hyaluronidase. As a matter of fact, Coburn(14,15) advocated plasma levels of at least 25 mg per 100 cc to secure prompt and progressive subsidence of the inflammation. This concentration usually makes the patient toxic with tinnitus, nausea, etc. Recently, Fulton *et al.*(16) reported that concentrations of sodium salicylate up to 100 mg % failed to inhibit hyaluronidase *in*

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vitro. In order to obtain only a slight effect of this enzyme in our experiments, we were forced to raise the salicylate concentration ten times higher than Fulton by using 10 mg of sodium salicylate per cc which is beyond the range of therapeutic dosage. In view of recent clinical investigations,(17) there is a possibility that the modus operandi of salicylates is a stimulation of the adrenal glands liberating a hormone which relieves the rheumatic manifestations. Sodium gentisate, which is a metabolic product of salicylate is even weaker. In the study of 4-amino salicylic acid (PAS), the anti-hyaluronidase activity approaches that of ortho cresol with, however, a higher therapeutic index.

Of the utmost importance is the fact that neither benzoic acid nor para aminobenzoic acid inhibit hyaluronidase, whereas their hydroxyl derivatives do.

Summary. The anti-hyaluronidase activity of phenol substitution products increases markedly with the length of the carbonic side chain. Salts of aromatic acids, like sodium salicylate, have an activity of a lower order as compared with that of phenols with long carbonic side chain. A moderate increase in activity is also obtained by substituting chlorine, nitro or amino groups.

As the data indicate, there exists in general, a parallel course of the inhibitory power of the substances and their phenol coefficient.

We wish to thank S. Joffe for valuable laboratory assistance.

Both the enzyme and substrate used in these experiments were obtained from the Schering Corporation.

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Synergistic Action of Estrogenic Hormone and X-rays in Inducing Thymic Lymphosarcoma of Mice.* (17522)

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Lymphocytic neoplastic disease was induced by estrogens(1) or x-rays(2) in mice of certain genetic constitution when these agents were given in appropriate doses. The carcinogenic hydrocarbons are another group of lymphoma-inducing agents.(3) Synergistic or additive lymphomogenic effects of x-rays and the carcinogenic hydrocarbon, methylcholanthrene, were demonstrable(4) providing lymphoid tissue of the test animals was susceptible to the neoplasia-inciting action of each of the agents used independently.(5) In the present experiment the synergistic action of x-rays and estrogens was investigated. It had previously been demonstrated that the CBA and Bagg albino stocks of mice are susceptible to the induction of lymphomas by the action of either estrogens or x-rays.(6) A single dose of 200 r of x-rays is a minimum inducing dose; weekly injections of 5 micrograms of estradiol dipropionate for 14 weeks

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