In another experiment with seven-day-old White Leghorn chicks, the following results were obtained: (1) Each of twelve strains of virulent organisms was inoculated subcutaneously in one chick; all twelve chicks died within 24 hours. (2) Twelve chicks, injected intraperitoneally with 200 units of antitoxin two hours previously, were inoculated subcutaneously with the same twelve strains; all twelve chicks remained well for two weeks or longer. (3) Of twelve chicks inoculated with twelve different strains of avirulent *C. diphtheriae,* all but one remained well for two weeks. (4) Of ten chicks inoculated with ten different cultures of diphtheroids, all remained well.

The protocol of a similar experiment with strains other than those mentioned above is shown in Table 1.

TABLE 1

EFFECT OF VARIOUS STRAINS OF CORYNEBACTERIA IN CHICKS

Organism	Result of virulence test in rabbit	Strains	Chicks	Dead	Paralyzed	No visible reaction
C. diphtheriae (with antitoxin)	Positive	10	20	4†	0	16
C. diphtheriae (no antitoxin)	Positive	10	20	19	1	0
C. diphtheriae	Negative	10	20	1*	0	19
C. xerosis	Negative	10	20	1	0	19
C. pseudodiphthericum	Negative	10	20	0	0	20

† The other chick of these pairs, in each instance, survived with no visible illness.
* Only after 5 days; no paralysis observed.

Two White Leghorn chicks were inoculated with each strain. The results are essentially the same as those obtained in the previous experiments.

Altogether, of fifty unprotected chicks injected with 35 different strains of rabbit-virulent *C. diphtheriae,* only one survived, and this after a severe illness, while, of 38 antitoxin-protected chicks injected with the same strains, 33 survived and 5 died, 3 of them from nonspecific causes. Of 36 chicks injected with 26 strains of avirulent *C. diphtheriae,* 34 survived and 2 died. In addition, of fifty chicks receiving thirty strains of diphtheroids, both *C. xerosis* and *C. pseudodiphthericum,* all but one survived with no apparent illness. The possibility of using chicks as test subjects in determining the virulence of cultures of *C. diphtheriae* is thus clearly indicated.

Results obtained with toxic filtrates were equally definite. A preliminary experiment has shown that chicks succumb to *intraperitoneal* injection of as little as 0.5 guinea-pig-M.L.D. of toxin. In Table 2 details are given concerning a group of 48 chicks receiving five different doses of toxin. The data indicate a susceptibility approximating that of 250-gram guinea pigs. In those chicks designated as "ill," paralysis of the wings and legs was a prominent feature. The chicks given doses of toxin larger than 1.0 M.L.D.

TABLE 2

EFFECT OF VARIOUS DOSES OF DIPHTHERIA TOXIN UPON SEVEN-DAY-OLD WHITE LEGHORN CHICKS

Dose of toxin (guinea pig M.L.D.s)	Chicks injected	Fate
3.0	6	6 dead within 26 hours
1.5	12	11 dead within 40 hours 1 very ill for 36 hours, survived, later showed paresis
1.0	13	6 dead within 72 hours 3 " " 5 days 2 ill for 2 days, survived, later showed paresis 2 no evident illness
0.5	13	2 dead within 72 hours 3 " " 5 days 2 moribund and killed on fifth day 1 dead after 6 days 3 ill for 2–3 days, survived, later showed paralysis 2 no evident illness
0.1	6	3 slightly ill up to 96 hours, survived, no damage 3 no evident illness

died promptly. Those receiving less than 1.0 M.L.D. also sometimes died or were ill, but the effect of the toxin was definitely delayed, and paralysis was sometimes not manifest for 36 hours. The contrast between 1.0 M.L.D. and 0.5 M.L.D. in this respect, for example, was quite evident. The reaction to a given dose seemed to be more uniform in the chick than is the case with guinea pigs.

Thus, it has been shown that seven-day-old White Leghorn chicks succumb regularly within 48 hours to subcutaneous injections of 48-hour infusion-broth cultures of strains of *C. diphtheriae* which are virulent for rabbits. The chicks are rarely, if at all, affected by similar infections if protected by intraperitoneal injections of at least 200 units of antitoxin given 2 hours before. Cultures of *C. diphtheriae* and of diphtheroids, previously shown to be avirulent for rabbits, have no perceptible effect on the chickens.

Further, it has been demonstrated that week-old White Leghorn chicks have about the same degree of susceptibility to diphtheria toxin as 250-gram guinea pigs.

These experiments clearly indicate the value of the chick in the study of the diphtheria group of organisms. It is possible that both virulence tests and tests for the potency of diphtheria toxin can be satisfactorily made in week-old chicks. The economic advantages are obvious.[6]

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THE ANTI-TRYPTIC PROPERTIES OF HEPARIN

INVESTIGATIONS of the possible prolongation of insulin action by inhibitors of proteolytic activity[1] have

[6] The author takes pleasure in acknowledging, with appreciation, the technical assistance of Dr. Elizabeth I. Parsons and the advice and encouragement of Dr. Thomas B. Turner.

led to the discovery that heparin is a trypsin inhibitor. This resulted from the examination of many compounds in a search for a simple substance which might retard the activity of specific proteases.

Crystalline trypsin inhibitor is a powerful anti-protease, but the preparation of this compound is time-consuming and the yields comparatively small, hence the search for a substitute. Blood plasma, while a good source of anti-protease activity, is too complex a mixture to permit a carefully controlled study, and the yield of trypsin-inhibitor which may be isolated from plasma[2] is apparently only a small fraction of its total inhibitory power. Similarly, the amino-acid concentrate of Northrop[3] as well as the anti-trypsin of egg white of Balls and Swenson,[4] while comparatively simple to prepare, did not lend themselves easily to further purification in our hands. Heavy metals as well as certain basic dyes are strongly anti-tryptic, but the addition of these substances to the proteases caused the latter to be precipitated from solution, a phenomenon which might easily explain the anti-protease activity.

Many basic dyes are powerful anti-coagulants,[5] and our observation that they may inhibit proteolysis suggested a relationship between the two processes. It was therefore logical to proceed to an investigation of heparin.

Table I presents representative data of the effects of heparin on the hydrolysis of casein by trypsin and

TABLE I

EFFECT OF HEPARIN ON [illegible] IN BY TRYPSIN AND CHYMOTRYPSIN AT [illegible] ATION OF 3 ML SAMPLES OF [illegible] EXPRESSED IN [illegible] (N. [illegible])

Added to 25 cc of 6 per cent. casein after 30-minute contact	Time in minutes						
	15′	30′	60′	120′	180′	300′	480′
1 ml trypsin 1 ml H_2O	.01	.06	.14	.20	.33	.43	.53
1 ml trypsin 1 ml heparin	.00	.00	.04	.07	.13	.20	.29
1 ml chymotrypsin 1 ml H_2O	.05	.11	.18	.25	.31	.39	.48
1 ml chymotrypsin 1 ml heparin	.04	.11	.19	.25	.30	.39	.48

chymotrypsin. These proteases were isolated from beef pancreas and recrystallized according to the techniques described by Northrop and Kunitz.[6] The heparin[7] was prepared to contain 2 mg per ml of water. The pH of this solution was 7.30. The casein (Hammarsten) was dissolved in sufficient sodium hydroxide to give a final pH of 7.5 and diluted to a concentration of 6 per cent. The enzyme solutions used contained approximately 0.03 mg of protease nitrogen per ml. The latter were carefully titrated with phosphate buffer to pH 7.30, using a standardized glass electrode system. No inhibition of trypsin is obtained unless the heparin is allowed to remain in contact with the alkaline trypsin solution for about thirty minutes before the two are added to the casein. This fact is particularly significant, since a parallel relationship obtains with trypsin and trypsin inhibitor.[6] Furthermore, the heparin-trypsin addition complex like the inhibitor-trypsin compound may be dissociated by acidifying to pH 3.0 for about 30 minutes to recover all the tryptic activity.

The data in Table I clearly indicate a marked inhibition of trypsin, especially during the first hour of digestion, but no apparent inhibition of chymotrypsin by similar concentrations of heparin. This effect has been checked many times by nephelometric as well as refractometric techniques to eliminate the possibility that the inhibition might be an artifact of the formol titration. Since trypsin catalyzes the clotting of recalcified oxalated plasma, and chymotrypsin is comparatively inert in this respect, the anti-tryptic propensity of heparin acquires additional significance. Preliminary experiments have indicated that in a given sample of plasma, added trypsin and heparin are mutually antagonistic and that clotting will not occur unless the amount of trypsin added is more than enough to neutralize the effect of heparin. Ferguson[8] has postulated the presence in blood of a "thromboplastic enzyme" which hypothesis is supported by the above observations.

Alterations in the anti-tryptic titre of plasma have been unexplained phenomena for several decades. Having the information that heparin is anti-tryptic, it may now be possible to correlate certain increases in blood anti-trypsin with increases in heparin. For example, Jaques and Waters[9] have recently shown that heparin is greatly increased in the blood of dogs in peptone shock, whereas in 1915 Jobling, Peterson and Eggstein[10] showed that peptone shock caused an increase in blood anti-trypsin. Further research on the amounts of heparin in various physiological states may present similar correlations.

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