THE ANTHRACIDAL SUBSTANCE IN THE SERUM OF WHITE RATS.*

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METCHNIKOFF,[1] more than 20 years ago, developed his theory that the property of digestion in ameboid cellular organisms is possessed also by certain cells of higher animals. The part of the phagocytes in resisting bacterial invasion he holds to be an important one, although not necessarily in every case the only means of defense; but in immunity to anthrax bacilli he gives to phagocytosis an important place, and a large part of his early experimental work was done on anthrax bacilli and spores.

When Behring,[2] a few years later, discovered the enormous power of the serum of the white rat to destroy anthrax bacilli, he concluded that this alone was responsible for the natural immunity which this animal possesses. There are, however, other cases that are not so easily explainable; the dog, for instance, is immune to anthrax infection, and yet its serum furnishes an excellent culture medium for the germs *in vitro;* while the rabbit, on the other hand, which is very susceptible, possesses a serum as strongly bacteriolytic in the test tube as that of the rat itself.

Behring explained the anthracidal power of the rat serum as due to alkalinity, for the following reasons:

1. Rat serum has a higher alkalinity than other sera.

2. Anthrax germs will not grow in it.

3. Through addition of acids to neutralization the serum becomes an excellent medium for anthrax.

4. Injections of oxalic acid into rats during life cause the serum to lose its anthracidal power and rob the animal of its resistance.

About a year ago Pirenne[3] also came to the conclusion that the anthracidal power of rat serum, contrary to what is true of most immune sera, is due to one body which probably is an organic, basic substance. If the anthracidal power is due to the interaction of

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[1] *Virch. Archiv*, 1884, 96, p. 502.

[2] *Ges. Ahh.*, Leipzig, 1893.

[3] *Centralbl. f. Bakt.*, 1905, 36, p. 256.

two bodies, complement and amboceptor, the serum should possess the properties ordinarily ascribed to bacteriolytic sera in general. The amboceptor being a stable body, and highly resistant to all influences, its presence is difficult to disprove; but complement is a labile substance, easily destroyed, whose presence or absence is readily shown. Now, the following are the characteristics of the anthracidal substance in rat serum, according to Pirenne: It is thermostable, resistant to 64° C.; its anthracidal action is lost on neutralization of the serum; it does not give rise to a specific antibody in guinea-pigs; it resists filtration, and also exposure to sun-light for 15 days; it keeps for 230 days on ice, and its action is not suspended at 0° C. For these reasons Pirenne concludes that complement in the usual sense takes no part in destruction of anthrax bacilli by the serum of the white rat.

I have repeated Pirenne's experiments, and while my results agree with his in many respects, there are important points of difference. The blood for these experiments was drawn by means of a syringe directly from the heart, under aseptic precautions, and allowed to clot against the side of a sterile glass vessel. With old rats chloroform was not given except in a few cases, as the rats could be handled by means of a towel until firmly tied down on a small board. About 4 or 5 c.c. of blood could be drawn without killing the rat, if intraperitoneal injections of salt solution were immediately given. The clear serum separating off was tested undiluted, and also diluted one part in four of normal salt solution, and as the anthracidal power was found not to be diminished by this dilution, this was then used in all subsequent tests for comparative purposes, in order to prevent coagulation on heating the serum. Heating was done in a water bath, and continued in each case for 40 minutes. In each experiment the different tubes contained the same number of drops of serum. They were inoculated with one loopful of an anthrax suspension made by rubbing up a 24 hour agar slant culture in 3 c.c. of NaCl solution, one loopful from each tube being plated at once. After four hours at 37° C. one loopful from each tube was plated again, and the colonies that developed were counted at the end of 24 hours at 37° C. An amount of broth equal to the amount of serum in each tube served as a control.

Table 1 illustrates the results obtained in these experiments.

We see that the serum of old rats (not chloroformed and not previously bled) strongly resists any temperature under 66° C., and even at this point the lytic power is only half destroyed. After heating at 68° C., the serum allows complete growth, as compared with broth. This result was obtained in numerous instances.

TABLE 1.

DESTRUCTION OF ANTHRAX BACILLI BY THE SERUM OF THE WHITE RAT.

	ONE LOOP IN EACH PLATE	
	At Once	4 Hours
Normal rat serum	13	0
" " " heated at 58°C	3,144	1
" " " " " 60	3,144	40
" " " " " 62	3,144	60
" " " " " 64	3,144	176
" " " " " 66	2,800	440
Control (Broth)	3,144	∞

In the normal unheated serum of white rats a massive destruction of bacilli appears to occur practically at once, as shown by the insignificant number of colonies that develop in the plates immediately after mixing the bacilli with the serum as compared, with the number in the plates from the control tubes of broth.

Results similar to those recorded in Table 1 were obtained by plating the entire contents of the tubes, the number of bacilli inoculated being determined by plating one loop from the anthrax suspension used for inoculating the serum. In this series also it was found that the anthracidal power of the serum is seriously impaired or completely lost only on heating at 66° and 68° C. for 40 minutes. As a general rule 0.2 c.c. of normal serum suffices to kill from 9,000 to 10,000 bacilli in four hours. Chloroform anesthesia and previous bleeding of rats appear, as was shown by Behring, to reduce somewhat the anthracidal power. All attempts at reactivating heated serum by means of serum exhausted of anthracidal substance by treatment with large quantities of anthrax bacilli were unsuccessful. In certain experiments by Hektoen and Ruediger[1] on the antilytic action of salts and other substances the serum of the white rats employed was found to have lost its bactericidal power on heating at 58°C. for 30 minutes.

[1] *Jour. Infect. Dis.*, 1904, 1, p. 379.

In endeavoring to explain the discrepancy between the last-named results and those of Behring, Pirenne, and myself, the thought occurred that possibly the age of the animals might play some rôle. In order to determine the actual facts, the sera of 12 rats between the ages of three weeks and five months were tested, and it was found that the younger the rat the more thermolabile its serum, as shown in Table 2, where a summary of these experiments is given. Inquiry revealed the fact that Hektoen and Ruediger in their work used young rats.

TABLE 2.

RELATION OF AGE OF THE RAT TO THE THERMOLABILITY OF ITS SERUM.

Age of Rats	Loss of Anthracidal Power Begins on Heating at	Anthracidal Power Completely Lost on Heatfng at
3 weeks	56°C.	58°C.
1 month	56	58
2 months	56	58
3 "	56	60
5 "	58	62
Ö t	60	66

Whether the thermolability of the serum of young animals is due to the presence of complement and amboceptor early in the life of the white rat, or, as is more likely, to a smaller quantity of the same substance present in the older animals, has not been determined on account of lack of suitable animals.

Behring showed that white rat serum is more alkaline than other sera which are not destructive to anthrax; that neutralization destroys this anthracidal property; and that injections of oxalic acid during life reduce the serum to a good culture medium by reducing, so he concluded, the alkalinity. From these observations he inferred, as already pointed out, that the anthracidal action is due to alkalinity, and in this opinion he is supported by Pirenne. There are, however, certain difficulties in the way of accepting this conclusion. Thus I have found that human serum, which permits of a fairly ready growth of anthrax bacilli, is not less alkaline than rat serum. Although unquestionably anthracidal action is lost on neutralization, it is possible that the action may be due to effects other than neutralization of the reaction. Pirenne, although he agreed with Behring, found, on heating the serum to 56°—a point where the

anthracidal action was unimpaired—that the alkalinity was reduced one-quarter. I have not been able to confirm Pirenne's observations. Titrating serum, heated at 58°, 60°, and 70° C. for 40 minutes against $\frac{n}{100}$ oxalic acid, using rosolic acid as indicator, showed, in my hands, no reduction of alkalinity as compared with normal serum (Table 3). Now the fact that serum deprived of all anthracidal power by heating at 70° C. shows no loss of alkalinity suggests strongly that the anthracidal action, after all, depends on other factors—single or combined—than alkalinity, in spite of the circumstance that the serum loses its anthracidal action on neutralization by acids.

TABLE 3.

THE EFFECT OF HEAT ON ANTHRACIDAL POWER AND ALKALINITY OF RAT SERUM.

SERUM	ALKALINITY	NO. OF BACILLI IN TOTAL QUANTITY	
		At once	4 Hours
Normal serum	1 c.c. = 0.0014 NaOH	8,000	0
Serum heated at 70° C.	1 c.c. = 0.0014 "	8,000	∞

Repeated injections of guinea-pigs with increasing doses of defibrinated rat blood, from 3 to 5 c.c., did not give rise to any substance that neutralized the anthracidal body in the rat serum.

While the serum of a rat five months old was deprived of its anthracidal power on exposure to direct sunlight for four days, the serum of old rats resisted this exposure longer, but not so long as in Pirenne's experiments.

It is of interest to note that the opsonic power of rat serum on anthrax bacilli, human leucocytes being used as phagocytes, closely follows its anthracidal power as regards resistance to heat, being fully destroyed only after heating at 66° C. for 30 minutes; neutralization with oxalic acid, however, has no destructive effect upon the opsonin.

CONCLUSIONS.

1. While the anthracidal substance in the serum of adult white rats, studied by Behring, Pirenne, and others, is thermostable, being destroyed by heating at 68° C. for 30 minutes, the anthracidal substances in young rats is less resistant to heat and is completely destroyed at 58° C. in rats under two months old.

2. While neutralization of rat serum with oxalic acid destroys its anthracidal power, as first pointed out by Behring, heating the serum to 68° to 70° C., at which temperature its anthracidal property is lost, does not reduce its alkalinity. Consequently the inactivation by oxalic acid would seem to depend upon more complex changes than mere reduction of alkalinity, and it also would seem likely that the anthracidal power of rat serum is not dependent on alkalinity only.

The normal serum of the white rat contains an opsonin for anthrax bacilli that is thermostable, like the bactericidal substance, but this opsonin is not affected by neutralization of the serum with oxalic acid.