

CHEMO-IMMUNOLOGICAL STUDIES ON LOCALIZED
INFECTIONS.*

THIRD PAPER: SOME FURTHER OBSERVATIONS UPON THE ACTION
OF CERTAIN SOAPS ON THE PNEUMOCOCCUS AND ITS
EXPERIMENTAL INFECTIONS.

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As related in the first paper,¹ it has been found that sodium oleate injures, kills, and dissolves virulent pneumococci, and although its action in the test tube is inhibited by serum, it is possible under certain quantitative conditions of experiment to circumvent this serum inhibition by the use of boric acid and thus to employ successfully sodium oleate and immune serum conjointly in combating artificial pneumococcic infections. It was also stated that after inoculating susceptible animals with cultures of the pneumococcus, better results, in preventing fatal infection, were secured by treatment with combined sodium oleate and pneumococcus immune serum than with either alone. At the same time it was pointed out that there is a limit in the time which can be allowed to elapse between inoculation and treatment beyond which it has not been found possible to save the infected animal, although the treatment too long delayed to prevent death, usually defers the fatal issue.

Realizing, therefore, the limitations to the use of sodium oleate and yet recognizing in it an agent which possesses in some degree the three most important desiderata of an "internal" antiseptic remedy, *viz.*, great toxicity for the infecting parasite, little toxicity for the host, and ability to act in the presence of serum,—we have continued the studies in combating artificial pneumococcic infections in two directions. The one had for its object a widened field

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¹Lamar, *Jour. Exper. Med.*, 1911, xiii, 1.

of the therapeutic application of sodium oleate and immune serum, as well as the enhancing of the activity of the soap in the presence of serum; the other, a search for a more effective agent to substitute for sodium oleate. This paper deals with the latter phase of the problem, yet it may be said here that some encouraging results, to be published later, have been obtained in respect to the extension of the therapeutic use of combined sodium oleate and pneumococcus immune serum.

Comparing the strong bactericidal action of the soaps of oleic acid with the absolute inactivity of those of its analogue in the saturated series—stearic acid—we have in the fact of unsaturation and resulting chemical affinities a probable explanation of this activity and hence an indication of the direction in which the search for a substitute might well be begun, *i. e.*, among the more highly unsaturated fatty acids. Several such acids were obtained and tested as free acids and as alkali soaps. A summary report of their action on the pneumococcus in the test tube was made in the second paper,² where it was stated that potassium linoleate and linolenate were much more destructive to the pneumococcus than oleates and, also, that a given quantity of serum inhibited their action to a lesser degree. The present paper is concerned with the properties of these two acids.

Kahlbaum's linoleic and linolenic acids were used. The former had physical properties and an iodine value (181.4) indicating approximate purity; the latter an iodine value of only 243.2, indicating admixture of less unsaturated acid, partial "drying," or both. Yet considering the impossibility of obtaining the acid in a pure state and the great difficulty of obtaining it more nearly pure than Kahlbaum's, it was considered a satisfactory specimen for the purpose of this investigation. Potassium and sodium soaps were made in the usual way with alcoholic alkali solutions. The soaps are more freely soluble than the corresponding oleates and form in 10 per cent. concentration perfectly clear, limpid solutions. No difference was detected in the action of the potassium and sodium soaps of the same acid, and a difference only in intensity of action between the soaps of the two acids, the linolenate being about one half again as

² Lamar, *Jour. Exper. Med.*, 1911, xiii, 380.

potent as the linoleate. The comparative bacteriolytic strengths of oleate, linoleate, and linolenate may be expressed roughly by the ratios 1:4:6.

When the linoleate or linolenate soaps are added to a broth culture of pneumococcus so as to form a dilution of 1 to 1,000, and even 1 to 5,000, the pneumococci are killed and dissolved with great rapidity. At dilutions of 1 to 500 and 1 to 1,000 the rapidity and intensity of action are such that, as revealed by hanging drop and film preparations, the solution of the cocci is well under way in one minute and complete in a few minutes. With linoleate at 1 to 4,000 and linolenate at 1 to 6,000, the culture is sterilized and the cocci apparently completely dissolved within one hour at room temperature. When the dilution of soap is greater, solution of the cocci is correspondingly less, although it is still manifest at dilutions of 1 to 20,000 of linoleate and 1 to 30,000 of linolenate.

In a similar manner to that of sodium oleate, highly dilute solutions of these soaps change the pneumococci in some way, without killing or appreciably altering the form or staining properties, so that when washed and resuspended in salt solution or serum they disintegrate with great rapidity. In normal serum the disintegration is incomplete, and the cocci which escape destruction multiply later. In immune serum destruction is complete. This kind of injurious action is exerted by sodium linoleate in dilution of 1 to 60,000 and by sodium linolenate in dilution of 1 to 100,000.

Moreover, when pneumococci are so treated with the great dilutions of the soaps, washed and resuspended in salt solution, and injected alone and in combination with normal and immune serum into rats, all the animals die of pneumococcic septicemia except those which received the treated cocci and immune serum. The latter are at most transitorily ill. A protocol will serve to illustrate one of the experiments.

March 30, 1911. 12:30 P. M. Treated a 22 hours' plain broth culture of N. I. 66 with sodium linolenate at a dilution of 1 to 75,000 for one hour at room temperature; collected the bacteria and washed once with salt solution by centrifugalizing; resuspended the cocci in salt solution so that 0.5 c.c. of suspension represented 2 c.c. of the original culture. Washed and resuspended in the same way the normal diplococci contained in another portion of the same broth culture.

3 P. M. Injected white rats, in duplicate series, intra-abdominally; each rat receiving 0.5 c.c. of suspension or the equivalent of 2 c.c. of a culture of which 0.000001 c.c. is fatal for grown rats.

TABLE I.

	Mar. 31, 1911.	Result.
No. 1. Soaped cocci alone.	Rather ill.	Died 40 hrs. after inoculation; pneumococcus septicemia.
No. 2. Soaped cocci alone.	More ill.	Died 38 hrs. after inoculation; pneumococcus septicemia.
No. 3. Soaped cocci + normal serum, 0.2 c.c.	Very ill.	Died 36 hrs. after inoculation; pneumococcus septicemia.
No. 4. Soaped cocci + normal serum, 0.2 c.c.	Very ill.	Died 60 hrs. after inoculation; pneumococcus septicemia.
No. 5. Control, washed cocci + immune serum, 0.2 c.c.	Moderately ill.	Died 60 hrs. after inoculation; pneumococcus septicemia.
No. 6. Control, washed cocci + immune serum, 0.2 c.c.	Moderately ill.	Died 82 hrs. after inoculation; pneumococcus septicemia.
No. 7. Soaped cocci + immune serum, 0.2 c.c.	Apparently well.	Never noticeably ill. Discarded, well, on May 10, '11.
No. 8. Soaped cocci + immune serum, 0.2 c.c.	Apparently well.	Never noticeably ill. Discarded, well, on May 10, '11.

SERUM INHIBITION.

Blood serum inhibits the bactericidal (and hemolytic) action of soaps. But the effect is not exhausted by this simple observation, for there may be recognized all degrees of inhibition from slight to complete, according to the ratio of quantity of serum to concentration of soap. Further, a given quantity of serum, but one not in excess, inhibits the action of linoleates and linolenates much less than of oleates. For instance, 0.1 cubic centimeter (or 5 per cent.) of serum completely inhibits the action of 0.5 milligram of sodium oleate, yet only slightly retards the dissolving of blood cells and pneumococci by the same quantity of linolenate, and does not quite completely check the action of only one fifth as much.

Hence we were led to inquire into the quantitative relations of serum inhibition and the comparative affinities of the several soaps for serum. Advantage was taken of the already mentioned similarity between the hemolytic and bacteriolytic reactions and a few hemolytic tests were made in order to bring out more clearly slight differences in the degree of lytic action. The next table illustrates the quantitative relations in respect to the inhibition of lysis, existing between serum and the different soaps.

June 6, 1911. Each tube contains 0.3 mg. of soap (dilution of 1 to 10,000); varying quantities of serum; 2 c.c. of a 5 per cent. suspension of dog washed blood cells; with salt solution to a total volume of 3 c.c. The results are expressed in terms of hemolysis after one hour in the incubator and twenty hours in the ice chest.

TABLE II.
Hemolysis.

	1 hour.			20 hours.		
	Sodium oleate.	Sodium linoleate.	Sodium linolenate.	Sodium oleate.	Sodium linoleate.	Sodium linolenate.
1. Control, soap alone.		All complete	in a few	minutes.		
2. Soap, 0.3 mg.; serum 0.05 c.c.	Great	Complete	Complete	Complete	Complete	Complete
3. Soap, 0.3 mg.; serum 0.1 c.c.	o	Great.	Great.	o	Complete	Complete
4. Soap, 0.3 mg.; serum 0.2 c.c.	o	Moderate.	Great.	o	Complete.	Complete.
5. Soap, 0.3 mg.; serum 0.3 c.c.	o	?	Moderate.	o	Slight	Complete.
6. Soap, 0.3 mg.; serum 0.4 c.c.	o	o	Slight	o	o	Slight.
7. Soap, 0.3 mg.; serum 0.5 c.c.		o			o	o
8. Soap, 0.3 mg.; serum 0.6 c.c.		o			o	o
9. Soap, 0.3 mg.; serum 0.7 c.c.		o			o	o
10. Soap, 0.3 mg.; serum 0.8 c.c.		o			o	o
11. Control, blood cells alone.		o			o	
12. Control, serum 0.4 c.c.		o			o	
13. Control, serum 0.6 c.c.		o			o	
14. Control, serum 0.8 c.c.		o			o	

Thus it is evident that a small quantity of serum only retards, and does not prevent, complete lysis; that to inhibit completely any hemolysis a definite quantity of serum must be present; and that the required quantity is greater for the linoleate than for the oleate, and still greater, in turn, for the linolenate.

Moreover, it has been found that if relatively concentrated solutions of any of these soaps are employed blood cells are dissolved and pneumococci killed in 50 per cent., and even in whole serum. This is shown in the following tables.

June 15, 1911. Horse blood is defibrinated and centrifugalized; the cells being resuspended in portions in 25 per cent., 50 per cent., and whole horse serum. To parts of each suspension a solution of each soap is added so as to make ultimate dilutions of 1 per cent., 0.2 per cent. and 0.1 per cent.

TABLE III.
Hemolysis.

	1 Hour.								
	25 per cent. serum.			50 per cent. serum.			Whole serum.		
	Oleate.	Linoleate.	Linolenate.	Oleate.	Linoleate.	Linolenate.	Oleate.	Linoleate.	Linolenate.
I per cent. soap.....	Complete.	Complete.	Complete.	Almost complete.	Complete.	Complete.	Great.	Almost complete.	Complete.
0.2 per cent. soap.....	Complete.	Complete.	Complete.	Great.	Complete.	Complete.	Slight.	Moderate.	Moderate.
0.1 per cent. soap.....	Trace.	Complete.	Complete.	0	Trace.	Slight.	0	Trace.	Slight.
	20 Hours.								
I per cent. soap.....	Complete.	Complete.	Complete.	Complete.	Complete.	Complete.	Complete.	Complete.	Complete.
0.2 per cent. soap.....	Complete.	Complete.	Complete.	Almost complete.	Complete.	Complete.	Slight.	Moderate.	Great.
0.1 per cent. soap.....	Moderate.	Complete.	Complete.	Trace.	Slight.	Moderate.	Trace.	Trace.	Slight.
Control, blood cells alone.....	0	0	0	0	0	0	0	0	0

June 17, 1911. Collected the pneumococci in a 24 hours' plain broth culture of No. I. 77 and resuspended portions in 50 per cent. and in whole horse serum to one fourth of the original volume of the culture. Then added solutions of the several soaps to portions of each suspension so as to make ultimate dilutions 1 per cent. and 0.2 per cent. The mixtures were permitted to remain one half hour at room temperature, after which transplantations from each to glucose serum agar were made.

The results are given in terms of growth of pneumococcus after 48 hours' incubation of the transplantations.

TABLE IV.
Bacteriolysis.

	50 per cent. serum.	Whole serum.
1. Control, suspension of cocci alone.	Profuse growth.	Profuse growth.
2. Sodium oleate, 1 per cent.	Sterile.	One colony.
3. Sodium oleate, 0.2 per cent.	Moderate growth.	Less growth than in control.
4. Sodium linoleate, 1 per cent.	Sterile.	Sterile.
5. Sodium linoleate, 0.2 per cent.	Slight growth.	Moderate growth.
6. Sodium linolenate, 1 per cent.	Sterile.	Sterile.
7. Sodium linolenate, 0.2 per cent.	Sterile.	Scant growth.

These observations, as well as the others that relate to the parallelism between the hemolytic or bacteriolytic activities and the quantity of serum required to inhibit them, lead to the belief that the action of the soaps of the unsaturated fatty acids is not due wholly to their power to dissolve lipoids but is due in part to their affinity for protein.

ATTEMPTS TO PREVENT SERUM INHIBITION.

The effort was made to prevent the serum inhibition of the lytic action of the more unsaturated soaps, as in the earlier experiments with sodium oleate. To this end, boric acid was employed first, since it was known to permit oleates to act in the presence of serum. The experiments, in test tubes, were made by adding boric acid in varying quantities to serum, then adding this mixture to the culture of pneumococcus, and then to the whole, sufficient soap solution to make an ultimate dilution within the limit required to produce complete solution of the diplococci in one hour in the absence of serum, *i. e.*, 1 to 4,000 of the linoleate and 1 to 6,000 of the linolenate. Film preparations and transplantations to glucose serum agar

served as indicators. Besides boric acid, a number of salts were tried: sodium borate, silicate, chlorid, citrate, and tartrate. The results were uniformly negative. The pneumococci in such mixtures were neither killed nor dissolved, except in the instance of sodium silicate, where the bacteria were killed, not dissolved, by the antiseptic action of this salt alone.

While these efforts to prevent serum inhibition in the test tube were not successful, it still remained to be seen whether the more unsaturated soaps would exert any influence in pneumococcus infections of the animal body. Experiments were made in which the soaps alone, and in various combinations with normal and immune serum and boric acid, were added to broth cultures and the mixtures injected at once into the peritoneal cavity of rats. Infection was prevented when the concentration of sodium linolenate was 1 to 1,000, a result which agrees with the extremely rapid lytic action of the soaps.

In other experiments, however, where the culture alone was injected first and immediately followed by injections of the soap solutions and the various mixtures of soap, boric acid, and serum, it was only in rare instances that the animals survived. And even then, the experiments being performed in duplicate series, the companion animals died.

In brief, it may be stated that although in the test tube, in the absence of serum, the more unsaturated soaps are much more potent in killing pneumococci, we have been unable so far to obtain with them in the animal body any results which compare at all favorably with those obtained by using sodium oleate. This may be explained by their much greater avidity for protein and their consequent rapid inactivation.

SUMMARY.

In the test tube, sodium linoleate and sodium linolenate kill and dissolve virulent pneumococci more rapidly and in higher dilutions than sodium oleate; otherwise their action is the same. The intensity of action on the cocci is directly proportional to the degree of unsaturation of the acid.

Blood serum inhibits the bacteriolytic and hemolytic action of unsaturated soaps partially or completely, according to definitive

quantitative relations between serum and soap. Small quantities of serum completely inhibit the action of dilute soap solutions, but even whole serum does not prevent bacteriolysis and hemolysis by relatively concentrated soap solutions, *i. e.* 1 per cent. and 0.5 per cent.

A given quantity of serum inhibits the action of the soaps in inverse ratio to their degree of unsaturation.

The hemolytic and bacteriolytic action of soaps of the unsaturated fatty acids is probably due in part to their avidity for protein and not wholly to their ability to dissolve lipoids.

Attempts to prevent serum inhibition of the lytic action of sodium linoleate and sodium linolenate were unsuccessful.

The more unsaturated soaps are not as efficient in preventing fatal pneumococccic infection of animals as sodium oleate.