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ON THE INFLUENCE OF THE REACTION AND OF DESICCATION UPON OPSONINS.[1]

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Whether the opsonic property of normal and immune serums, to which the writings of Wright and Douglas[2] have called renewed attention and endowed with special significance, is a new and hitherto undescribed property of serum or a special function of the usual immune bodies of serum is still a question of dispute.

Bulloch and Atkin,[3] Neufeld and Rimpau,[4] Barratt,[5] Neufeld and Töpfer,[6] Hektoen,[7] and Keith[8] share the view of Wright and Douglas that the opsonic action is due to the presence of certain hitherto unrecognized, distinct bodies, while Savtchenko,[9] Besredka,[10] and Dean[11] are inclined to consider the opsonins as identical with the amboceptors (fixators). Quite recently Muir and Martin[12] brought forth evidence that opsonization depends upon the coöperation of two substances, one of which, at least, closely resembles complement of serum in certain of its biological properties. Still later Levaditi and Inmann[13] asserted that opsonin are nothing but the complements of serum.

It is not my intention to enter into this discussion, but, for my purpose, it is necessary to mention that in common with complement, so-called, the opsonins are absorbed or fixed by sensitized bacteria, blood corpuscles, specific precipitates,[14] and indifferent

[1] Received for publication April 30, 1907.

[2] Wright and Douglas, *Roy. Soc. Proc.*, 1903, lxxii, 357; 1904, lxxiii, 128.

[3] Bulloch and Atkin, *Roy. Soc. Proc.*, 1905, lxxiv, 379.

[4] Neufeld and Rimpau, *Deut. med. Woch.*, 1904, xxx, 1458.

[5] Barratt, *Roy. Soc. Proc.*, 1905, lxxvi, 524.

[6] Neufeld and Töpfer, *Centralbl. f. Bakt.*, etc., 1905, xxxviii, 456.

[7] Hektoen, *Jour. of Infect. Diseases*, 1906, iii, 434.

[8] Keith, *Roy. Soc. Proc.*, 1906, lxxvii, 573.

[9] Savtchenko, *Ann. d. l'Inst. Pasteur*, 1902, xvi, 106.

[10] Besredka, *Ann. d. l'Inst. Pasteur*, 1904, xviii, 363.

[11] Dean, *Roy. Soc. Proc.*, 1905, lxxvi, 506.

[12] Muir and Martin, *Brit. Med. Jour.*, 1906, Pt. ii, 1783.

[13] Levaditi and Inmann, *Compt. rend. d. l. Soc. d. Biol.*, 1907, lxii, 725.

[14] Muir and Martin, *Brit. Med. Jour.*, 1906, Pt. ii, 1783.

bodies[15] as well as non-sensitized bacteria,[16] and exhibits thermolability and susceptibility to deterioration by age, similar to that which complement exhibits. Besides the liability to undergo adsorption and the high degree of thermolability, no other characteristics of ferments have been ascribed to opsonins. Were opsonins recognized as special ferments certain facts of theoretical and practical import should follow. Among other things we should look for an optimum of activity to be exhibited in the presence of a certain reaction which might or might not be that of the sample of serum which is being studied. If the degree of activity depended upon a given reaction then to obtain the optimum indication, upon which alone a measure of the quantity of opsonin could rest, this reaction would have to be secured. I have, therefore, made a study of the influence of the reaction upon the opsonic power of several blood serums for B. typhosus, B. dysenteriae, Streptococcus, and Staphylococcus aureus.

The results of these experiments harmonize so well that those alone relating to Staphylococcus aureus will be given in this paper.

THE INFLUENCE OF THE REACTION UPON OPSONINS.

The Influence of Alkalinity.—The degree of alkalinity of several serums were first titrated. Lacmoid paper was employed as indicator. The titration was conducted in the following manner. To 1 c.c. of serum $\frac{1}{20}$ N. solution of hydrochloric acid was gradually added until the reaction reached neutral. The amount of acid required for complete neutralization was taken as representing the degree of alkalinity of each serum. The resulting fluid was then made up to 5 c.c. with 0.9 per cent. salt solution. Thus the final mixture had a strength of one fifth of the original serum. By this means it was possible to prepare a series of mixtures, in which 0.5, 0.3, 0.2, 0.1 and null alkalinity were left unneutralized. These fluid mixtures were used for opsonization, and the results are given in Table I. The cross signs under each column represent the average number of the bacteria taken up by a phagocyte, each cross standing for three bacteria.

[15] Simon, Lamar and Bispham, *Jour. of Exper. Med.*, 1906, viii, 651.

[16] Bulloch and Atkin, *Roy. Soc. Proc.*, 1905, lxxiv, 379.

The bacteria were Staphylococcus aureus, twenty-four hours old, and the leucocytes human. The tubes were incubated at 37° C. for thirty minutes, and the technique employed was that given by Wright.

TABLE I.

	Varying Quantities of $\frac{1}{20}$ N. HCl Added to Leave the Indicated Degree of Alkalinity Unneutralized. Total Volume 5 c.c.					
	Original Alkalinity.	Alkalinity Left 0.5 c.c.	Alkalinity Left 0.3 c.c.	Alkalinity Left 0.2 c.c.	Alkalinity Left 0.1 c.c.	Neutral Reaction.
Dog	0.5 c.c.					
	+++	+++	+++	++++	++++	++++
Ox	0.75					
	++++	++++	++++	+++++	++++++	+++++++
Pig	0.8					
	+++++	+++++	+++++	++++++	+++++++	+++++++
Rabbit	0.65					
	+++	+++	+++	++++	++++	++++

As the foregoing table shows, a greater degree of opsonization is obtained at the neutral reaction than at the inherent alkaline reactions of the serums.

For the next series of experiments the inhibitory action of alkalinity upon opsonins was made more apparent by increasing the alkali in an ascending scale.

TABLE II.

	Original Alkalinity.	Amount of $\frac{1}{20}$ N. NaOH Added. Total Volume 5 c.c.				
		0	0.5 c.c.	1 c.c.	2 c.c.	3 c.c.
Dog	0.5 c.c.	+++	++	+	Negative	Negative
Ox	0.75	++++	+++	+	"	"
Pig	0.8	+++++	+++	+	"	"
Rabbit	0.65	+++	++	+	"	"

When 2 c.c. of $\frac{1}{20}$ N. sodium hydrate solution are added to 1 c.c. of a serum the opsonizing property of the latter completely dis-

TABLE III.

Neutralized Serum.	Amount of $\frac{1}{20}$ N. NaOH Added. Total Volume 5 c.c.					
	0	1.2 c.c.	1.4 c.c.	1.6 c.c.	1.7 c.c.	1.8 c.c.
Dog	++++	++	+	±	Negative	Negative
Ox	++++++	++	+	±	"	"
Pig	+++++++	+++	+	±	"	"
Rabbit	++++	++	+	±	"	"

appears; 1 c.c. of this solution added to 1 c.c. of serum reduces the opsonic activity to the minimum. Table III shows the maximum alkalinity in which opsonins are still able to act.

An alkali content approaching 1.6 c.c. suppresses the activity of opsonins. It is rather remakable that this degree of alkalinity is only about twice as high as that possessed by the majority of normal serums.

In Table I. it is shown that the activity of opsonin is greater at the neutral point than at the alkalinity possessed by normal serums, but the difference was not a marked one. In the following experiment the inhibitory influence of the native alkalinity is brought to light by means of dilution. The normal serum of the pig was neutralized with hydrochloric acid and divided into two portions. To one portion sodium hydrate solution was added to make it contain just enough alkali to reproduce the original degree of alkalinity, namely, 0.8 c.c. of $\frac{1}{20}$ N. To the second portion no alkali was added, but it was employed in the neutral reaction. These two portions were used for opsonization in ascending dilutions.

TABLE IV.

Dilution of the Neutralized Serum (Pig).	Alkalinity $\frac{1}{20}$ N. NaOH 0.8 c.c.	Alkalinity $\frac{1}{20}$ N. NaOH 0.8 c.c. Neutralized Back with $\frac{1}{20}$ N. HCl 0.8 c.c.	Control in Saline with Neutralized Serum Alone.
1:5	++++++	++++++	++++++
1:7	++++	++++++	++++++
1:10	+++	+++	+++
1:15	+	+++	+++
1:20	±	++	++
1:25	—	++	++
1:30	—	++	++
1:35	—	+	+
1:40	—	+	+
1:50	—	—	—
1:60	—	—	—
1:80	—	—	—

As Table IV. shows, the alkali restrained the opsonic action markedly and the minimum opsonization with the alkalinized portion was between 1 : 15 and 1 : 20, while the neutral portion was still active at 1 : 30 to 1 : 40 dilutions. This restraining effect of alkali was not so evident in the concentrated state of the serum, but was rapidly developed as the dilution was increased. It may

be remarked in passing that the inhibitory influence of the reaction on ferments is by no means a quantitative one. No matter how large a quantity of ferment is present in a mixture no action follows if the reaction is highly unfavorable. When the reaction is merely such as to inhibit partially, only so much effect is obtained as the degree of optimum permits. Under such circumstances, the greater portion of ferment may remain inactive. It is only when ferment finds itself in a fluid of optimum reaction that real quantitative differences can be manifested. The opsonic indices can seldom be driven beyond four, and usually not beyond two or three, in spite of repeated vaccination with bacteria. The reason for this limit is not at once apparent, but it is not improbable that even here the reaction of the serum may play a part. Judging from my experiments, the estimation of the opsonic index should be made at the neutral reaction and in a diluted serum. The advisability of dilution has been pointed out especially by Simon and his coworkers; and with this idea the results of Neufeld and Rimpau with certain immune serums agree.

The Influence of Acidity.—Opsonins having been shown to be more active in a neutral than in an alkaline medium, the next point to be examined was the influence of the acid reaction upon the opsonization. Table V shows that opsonins are highly sensitive to the acid reaction.

TABLE V.

Neutralized Serum.	Amount of $\frac{1}{20}$ N. HCl Added to the Neutralized Serum. Total Volume 5 c.c.					
	0	0.1 c.c.	0.2 c.c.	0.3 c.c.	0.5 c.c.	0.7 c.c.
Dog	++++	+++	++	+	±	Negative
Ox	+++++	+++	++	+	±	"
Pig	+++++	+++++	+++	+	±	"
Rabbit	++++	+++	++	+	±	"

If to the neutral serum is added 0.5 c.c. of $\frac{1}{20}$ N. hydrochloric acid solution, no opsonic action is to be obtained. The quantity of acid indicated in the table was added to an amount of the neutralized serum corresponding to 1 c.c. of the original.

Are Bacteria Opsonized in the Presence of Unfavorable Reaction? The restoration of opsonic activity.

Bacteria suspended in a serum which contains enough acid or alkali to suppress opsonization under ordinary conditions were centrifugalized and washed in 0.9 per cent. salt solution. The washed bacteria were then mixed with leucocytes and incubated as usual. It was found that these bacteria were not taken up by leucocytes. Thus it can be concluded that when the reaction is unfavorable, opsonins do not attach themselves to the bacteria. An analogous phenomenon was encountered by Hektoen and Ruediger[17] in the case of certain antiopsonic neutral salts.

In these respects, opsonins seem to differ from most bacteriolytic and hæmolytic amboceptors, for they sensitize the corresponding cells in a medium containing an inhibitory amount of acid for opsonins. The sensitiveness of opsonins to slight degrees of acid and alkali is similar to complement, although opsonins surpass complements in sensitiveness.

The fact was ascertained that inactivation of opsonins by means of an unfavorable reaction is a reversible reaction. If the excess of acid or alkali is removed by neutralization the opsonins become active once more. In this respect they resemble complements.[18] A difference is observed in that opsonins are more active in neutral media, and complements more active in alkaline media. Further calcium chloride reduces the action of complements but not of opsonins, provided the concentration of this chemical used does not exceed $\frac{1}{10}$ N.

Organic and inorganic acids in concentration of 1 N. render the serum opsonically inactive even after neutralization. The modification produced is irreversible.

Before leaving this topic, I wish to speak of an incident concerning the effect of small quantities of acids on the opsonic power of serum. At the beginning of this study, I employed $\frac{1}{40}$ N. solution of various acids—hydrochloric, nitric, sulphuric, formic, acetic, propionic, lactic, butyric, oxybutyric, citric, tartaric, oxalic, male-

[17] Hektoen and Ruediger, *Jour. of Infect. Diseases,* 1905, ii, 528.

[18] Noguchi, On the chemical inactivation and regeneration of complement. Read at the annual meeting of the Society of American Bacteriologists, Dec. 28, 1906.

inic, fumaric, itaconic, citraconic and glycerino-phosphoric—and as each in turn was added in small quantities to the serum the opsonic index was observed to rise. Contrary to my expectation, the addition of acid gradually but perceptibly increased the opsonic power of the serum. This first led me to think that acids might themselves act as opsonins. But the power was not further increased as the neutral point was reached, and from that point on, the increasing acidity finally suppressed it altogether.

EFFECT OF DESICCATION AND DRY HEAT ON OPSONINS.

Effect of Desiccation.—Opsonins are highly labile bodies. Their action disappears from the serum when it is allowed to stand for several days. In this respect they resemble complements. But no experiment has been recorded which tells us whether opsonins are obtainable in the dry state or not. I have tested this point. Normal serums of dog, ox and pig were dried at 23° C. with the aid of an air current, and the dissolved masses were tested again for their opsonic power. It was found that the opsonic power of the serum is not noticeably reduced by drying in this way. The next point was whether the opsonins would endure longer in the dried state. To determine this, I employed three samples of dried serum which had been preserved in the laboratory for two years. The serums were obtained from the rattlesnake, horse and ox, and were dried at a temperature approaching 48° C. Although I am unable to state the original activity at the time of drying, I can state that all three serums exhibited marked opsonic powers. Thus opsonins, once they are dried, are not labile substances.

On the other hand, dehydration of serum by means of alcohol renders the opsonins completely inactive. This fact would show that opsonins differ at least in this respect from many ferments, which stand treatment with alcohol, if not too prolonged.

Effect of Dry Heat.—The normal serum is robbed of its opsonic power by temperatures ranging from 55° to 60° C. Even immune serums, which are the most resistant in this respect, lost the greater part of their opsonic power at these temperatures, although traces may persist in a serum heated to 65° C. On the whole, opsonins are more thermostabile than complements, although less than the

usual immune bodies. The slight difference in the thermal resistance obtained by different investigators may depend upon differences in the reaction or some other physical or chemical conditions under which the tests were made.

Opsonins resemble ferments in their behavior to high temperature in the dry state. I have subjected the dried serums of ox, rabbit and dog to the temperature of 100°, 120°, 135° and 150° C., and then tested them for their opsonizing power. When the serums are heated to 120° C. and above, they become almost insoluble in water. To examine their activity, they must be powdered and emulsified in water. The bacterial emulsions were added to the former emulsions and the whole incubated at 37° C. for two hours. The result is given in Table VI.

TABLE VI.

	Opsonic Activity of the Heated or Unheated Dried Serum.					
	Unheated Dried Serum.	Dried Serum Heated to 100° C.	Dried Serum Heated to 120° C.	Dried Serum Heated to 135° C.	Dried Serum Heated to 150° C.	Unheated Dried Serum, Redissolved and then Heated to 56° C.
Ox serum	Active phagocytosis	Active phagocytosis	Active phagocytosis	Less active, but positive.	Very irregular occurrence of phagocytosis	Negative.
Rabbit serum (vaccinated)	"	"	"	"	"	"
Dog serum	"	"	"	"	"	"

From the foregoing experiment a high degree of resistance of the dried opsonins to high temperatures may be inferred. It is important to note that the dried serums regain their thermolability when redissolved in water, as is shown in the last column of the table.

Hence, it would appear that opsonins possess in common with ferments the property to resist in the dry state the action of high temperatures.

In the course of the experiments on desiccation and high temperature on opsonins, I took the opportunity of examining complements in the same manner.

It developed that complements do not disappear from the serum when it is dried at 23° C. The desiccated serum retained for several months complements unaltered in quantity and in activity.

Heating the dried serum to 100°, 120° and 135° C. does not deprive it of complementary action.

SUMMARY.

Opsonins reveal their maximum action in a medium of neutral reaction. No opsonization takes place in a serum which contains an amount of alkali corresponding to more than 1.6 c.c. of a $\frac{1}{20}$ N. solution, or acid more than 0.5 c.c. of this concentration per 1 c.c. of serum. Of several normal blood serums titrated (lacmoid used as indicator) the average alkalinity was found to be equivalent to about 0.8 c.c. of $\frac{1}{20}$ N. solution.

The opsonic index obtained in the native serums is not the expression of the action of the whole content of opsonins, but only so much as the degree of optimum of the reaction permits to come into action. Estimation of the opsonic power should, therefore, be made in a medium of neutral reaction and in diluted serum.

All serums have their opsonic power increased by diminishing the native alkalinity.

Opsonins whose activity is suspended by an unfavorable reaction become immediately active as soon as the reaction is brought back to the neutral point, unless the acid or alkali employed approaches the strength of 1 N., at which point the alteration becomes permanent.

Treatment of a serum with alcohol robs it of its opsonic power. The opsonic power of serum remains unaltered upon desiccation at 23° C. In the dry state opsonins are preserved for two years.

The temperatures of 100°, 120°, 135° and 150° C. do not destroy opsonins of the dried serum.

Complements of serum are also siccostabile and are preservable in that state for several months. Dry heat of 135° C. reduces but does not destroy the complementary power of the dried serum.

The opsonins and complements of the dried serum regain their original thermolability when they are dissolved in a proper amount of water.

Lastly, it may be emphasized that opsonins exhibit in their sensitiveness to reaction and resistance in the dry state to high temperatures certain properties characteristic of the ferments.