THE NATURE OF THE BACTERICIDAL SUBSTANCE IN LEUCOCYTIC EXTRACT.*

By WILFRED H. MANWARING, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research, New York.)

The phenomenon of phagocytosis furnishes evidence of the existence of powerful bacteriolytic substances within the cytoplasm of certain body cells. These endolysins are apparently quite distinct from serum bacteriolysins, since phagocytic cells are capable of destroying many bacteria that are not seriously injured by extracellular body fluids.

There is evidence that, under certain conditions, these intracellular lysins may be given off into the surrounding medium in sufficient quantities to play an important extracellular rôle. The automatic sterilization of old abscess cavities and the sterilization of the pneumonic lung could be accounted for by the assumption of the liberation of such endolysins as a result of cellular disintegration. This has given rise to the hope that eventually a valuable therapeutic agent may be obtained from phagocytic cells. It is conceivable that the therapeutic control of a number of infectious diseases, such as tuberculosis, pneumonia, and the various suppurations, may depend upon a knowledge of these substances. I have therefore undertaken to extend the present knowledge of these lysins, directing my initial effort to a determination of the approximate chemical nature of the bacteriolytic agent that can be extracted from leucocytes.

Considerable work has already been done with leucocytic extract.[1] Most investigators have studied rabbit leucocytes. These they have usually suspended in physiological saline, and have generally

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[1] For a bibliography and resumé of previous work on this subject, see Kling, Carl A., Untersuchungen über die bakterientötenden Eigenschaften der weissen Blutkörperchen, *Ztschr. f. Immunitätsforsch., Orig.*, 1910, vii, 1.

brought about the liberation of the bacteriolysin by repeated freezings and thawings, by heating the suspension to 50° C. for half an hour, or by simply allowing the suspension to undergo autolysis (table I).

TABLE I.

Bacteriolysin from Rabbit Leucocytes.

Twelve discarded rabbits were used as the source of the leucocytes. Each rabbit was injected in the right pleural cavity with 10 c.c. of a 5 per cent. suspension of aleuronat. The resulting pleural exudates were aspirated twenty-four hours later, added to an equal volume of 1.5 per cent. sodium citrate, and immediately centrifugalized. There were thus obtained about 10 c.c. of sediment consisting microscopically of a mixture of red and white cells in the approximate ratio of 1:1. No bacteria were seen, and samples transferred to agar gave no growth.

This sediment was washed twice by centrifugalization with physiological saline, was then suspended in four volumes of distilled water, and placed in the thermostat over night. In the morning the resulting extract was freed from cellular elements by centrifugalization, was heated to 58° C. for thirty minutes to destroy possible traces of serum bacteriolysin, and was then tested in various dilutions for its bactericidal power. In making these tests a loopful of an eighteen hour broth culture of *B. typhosus* was added to 1 c.c. of each fluid to be tested, and plates were made from the resulting mixtures at the times indicated. The table records the number of colonies on the plates thus obtained. The dilutions were made with fifth-physiological saline solution (p/5 sodium chlorid) to preserve osmotic relationships.

Material tested.	Time of plating.				
	1 min.	½ hr.	1¼ hrs.	3 hrs.	24 hrs.
Control, p/5 sodium chlorid	1,080	1,090	1,030	930	940
Undiluted extract	1	0	0	0	0
Diluted extract 1:2	20	0	0	0	3,000
Diluted extract 1:4	720	180	80	50	5,000
Diluted extract 1:8	1,240	1,100	1,200	1,340	1,000

The bactericidal agent thus obtained is relatively thermostable. It can be heated to 60° C. for an hour without loss of bactericidal power, and even to 80° C. without complete inactivation. This furnishes an easy means of differentiating this bacteriolysin from the serum bacteriolysin with which it may be mixed, since most sera are inactivated by heating to 55° C. for half an hour.

In addition to the thermostability, the most suggestive property of the extract for our present purposes is its loss of bactericidal

power on being passed through a Berkefeld filter (table II). From this it has been argued that the bacteriolysin is probably a colloid, presumably proteid in nature. Another suggestive property is the

TABLE II.

Effect of Filtration on Bacteriolysin.

Extract from rabbit leucocytes tested for its bactericidal power before and after its passage through a Berkefeld filter. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4 hrs.	24 hrs.
Original extract.....	600	0	0	0
Filtered extract.....	1,120	1,020	950	20,000

apparent insolubility of the active principle in ether, which has been taken to indicate that the bacteriolysin is probably not a simple soap or lipoid. The extract can be evaporated to dryness without loss of its bactericidal properties.

The greatest handicap to a more extensive chemical study of the extract has arisen from the small amount of substance with which previous investigators have worked. By injecting aleuronat into the pleural cavity of a rabbit, one seldom obtains more than a single cubic centimeter of leucocytes, which on extraction yields but a few milligrams of dried bacteriolysins. It was thought that this handicap might be overcome by using as the source of material some large animal. The horse was therefore selected for this work.

Rabbit Leucocytes.—As an introduction to the study of horse leucocytes, most of the previous work with rabbit leucocytes was repeated, and the properties outlined above were confirmed. The knowledge of the nature of rabbit leucocytic extract was extended somewhat. Thus, it was shown that for the purpose of the experiment it was immaterial whether the leucocytes were obtained under septic or aseptic conditions. After heating the extracts to 60° C., the extracts obtained under septic conditions and those secured aseptically were apparently identical. Most of the septic extracts, however, if unheated, rapidly lost their bactericidal properties, due presumably to bacterial overgrowth.

The number of red blood corpuscles mixed with the leucocytes

was found to influence the nature of the extract obtained. In the earlier experiments the strongest and most uniform bacteriolysins were obtained from leucocytes mixed with a relatively large number of red cells. So uniform was this relationship, that it suggested the possibility of the bacteriolysin being a product of the red cells, rather than a direct product of the leucocytes. This, however, was shown not to be the case, since equally strong bacteriolysins were subsequently obtained from leucocytes free from red blood corpuscles. The presence of red cells, however, influences the autolytic processes leading to the liberation of the endolysin. The exact nature of this influence has not yet been determined.

In the earlier experiments it was noticed that the leucocytic extracts, freed from cellular elements, usually lost their bactericidal powers on standing. After four or five days in the ice chest, most of the earlier extracts were inactive. It was found that this deterioration could be prevented by heating the extract to 58° C. for thirty minutes. This heating presumably leads to a destruction of proteolytic enzymes responsible for the deterioration.

It was found that the extract could be freed from hemoglobin without loss of bactericidal power by heating it to 62.5° C. for twenty minutes. When so heated, most of the hemoglobin is coagulated, leaving the supernatant fluid nearly colorless.

In working with a substance whose presence or absence must be followed throughout a series of chemical manipulations by tests of bactericidal power, it is necessary to control the osmotic properties at each stage of the manipulation. An osmotic pressure much above that of physiological saline (0.85 per cent. sodium chlorid) is injurious to many bacteria. It was thought that the easiest way to control osmotic relations would be to free the initial extract as completely as possible from crystalloids. This was done by dialysis. It was found that the extract from the rabbit leucocytes could be dialyzed free from sodium chlorid, and presumably free from most of the other crystalloids, without loss of bacteriolytic properties. Celloidin sacs were used as the dialyzing membranes.

Horse Leucocytes.—With this introduction the study of horse leucocytes was begun. These were obtained by intrapleural injec-

tions of aleuronat. It was found advisable in making these injections to observe the following precautions.

1. The material injected should be sterile, and free from irritating substances. The presence of irritating or infectious agents usually causes a rapid pouring out of fluid into the pleural cavity, and extensive diapedesis of red blood cells, but causes the liberation of comparatively few leucocytes.

2. The amount injected should not be too large. Too large an injection causes the formation of a layer of fibrin and aleuronat over the pleural surfaces, through which layer leucocytes apparently have difficulty in making their way. From 300 to 500 cubic centimeters of 5 per cent. aleuronat in 2 per cent. starch paste is a sufficient dose, the starch being added to prevent too rapid sedimentation.

3. The injection should be made in such a manner as to distribute the aleuronat widely throughout the pleural cavity. I usually insert the needle close to the vertebral column, in order that part of the aleuronat may pass to the mediastinal surfaces.

4. The resulting exudate should be aspirated at frequent intervals, to prevent degeneration.

Following such an injection, there are usually formed from one to three liters of pleural exudate daily, for the first week, the daily yield then decreasing and usually ceasing about the fifteenth day. The exudate usually contains about 5 per cent. of leucocytes, often quite free from red blood corpuscles.

The extraction of a bactericidal substance from these leucocytes offers considerable difficulty. Most of the methods which gave successful extracts with rabbit leucocytes were tried, but the extracts obtained were almost uniformly without bactericidal power. One reason for this initial failure is the great variation, not only in the leucocytes of different horses, but also in the leucocytes of the same horse on different days after the aleuronat injection. Horse leucocytes are also apparently very easily injured by manipulation, and easily influenced in their autolytic processes. The exact nature of these factors I hope to make clear in a later paper.

Most of the earlier successful extracts were obtained from un-

washed leucocytes, mixed with comparatively large numbers of red blood corpuscles. This gave rise to the following method of extraction, used throughout this paper.

The pleural exudate, drawn into about a tenth of its volume of 3 per cent. sodium citrate, was enriched by the addition of a sufficient amount of freshly drawn citrated horse blood to make the ratio between the red and white cells approximately 1 to 1. The cells were then thrown down by centrifugalization, washed once at 0° C. (to prevent agglutination) with 50 per cent. horse serum, suspended in about four volumes of distilled water, incubated at 35° C. for three hours, and then packed in ice over night. The next morning the resulting extract was freed from cellular elements by centrifugalization, heated to 58° C. for thirty minutes, and tested for its bactericidal power (table III).

TABLE III.

Bactericidal Extracts from Horse Leucocytes.

Eleven extracts from horse leucocytes prepared by the technique described above. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Extract 1	1,520	1,280	1,120	5,000
Extract 2	1,100	880	730	750
Extract 3	980	760	280	550
Extract 4	750	75	10	60
Extract 5	1,000	190	35	0
Extract 6	400	6	2	0
Extract 7	930	128	0	0
Extract 8	580	2	0	0
Extract 9	600	0	0	0
Extract 10	120	0	0	0
Extract 11	4	0	0	0

About two thirds of the extracts prepared by this method were found to possess fairly strong bactericidal properties. The bactericidal power, however, was usually less than that of the extracts from rabbit leucocytes. It is probable that further study of the method of extraction may lead to stronger and more uniform results.

The bactericidal substance in these extracts has apparently the same general properties as the bactericidal substance from rabbit

leucocytes. There are minor differences, as in thermostability and in relation to dialysis, to be presented in detail in the final paper, but the agreement throughout is sufficiently close to warrant the belief that in this extract also the bactericidal agent is probably a proteid. The question then arises as to the class of proteid to which it belongs.

Proteids are usually divided into classes according to their solubility or insolubility in distilled water and in various salt solutions. The process of salting out, in which certain salts are added to a solution in certain concentrations, furnishes an easy means of separating proteids of different classes. This process was applied to the leucocytic extract, but with results that at first were both inconsistent and contradictory and markedly inconstant.

Thus, in one experiment ammonium sulphate was added to the extract to saturation. A heavy precipitate formed immediately. This was thrown down by centrifugalization, washed once with saturated ammonium sulphate, redissolved in a small volume of distilled water, and dialyzed free from ammonium sulphate. The solution thus obtained was evaporated to dryness *in vacuo,* and tested for its bactericidal properties. Making no allowance for amounts lost during manipulation, the end-product was found to possess approximately half the bactericidal power of the initial extract.

This experiment was then repeated, adopting precautions to make the determination an accurate quantitative one. A larger initial volume of extract was taken, the precipitate was dissolved in a larger amount of distilled water, the centrifuge tubes and pipettes were carefully rinsed out and the rinse water was added to the solution to be dialyzed. The end-product of this more careful determination was without bactericidal properties.

Here are two determinations, on superficial examination identical,—one giving a strongly bactericidal end-product, the other an end-product without bactericidal powers. This indicates either that the technique was greatly at fault, or that there are complexities in the determination not sufficiently taken into account in the experimental method.

The only apparent difference between the two experiments is a

volumetric one. As a result of the attempt to make the second determination a quantitative one, the second precipitate was dissolved in a larger volume of distilled water. It was thought that the resulting differences in concentration might possibly make a difference in the subsequent process of dialysis. A study was therefore made of the effect of dilution on dialysis, with the discovery that dilution alone is sufficient to destroy completely the bactericidal properties of the extract within the time necessary for dialysis (table IV).

TABLE IV.

Effect of Dilution on Bacteriolysin.

Sample of a concentrated extract from horse leucocytes diluted with fifty volumes of distilled water and allowed to stand in the ice chest for forty-eight hours. The fluid was then evaporated to dryness and tested for its bactericidal power. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	2 hrs.	5 hrs.	24 hrs.
Diluted extract	920	720	700	1,200
Diluted extract 1: 2	940	820	740	1,100
Diluted extract 1: 4	930	750	770	1,200
Diluted extract 1: 8	900	720	730	800
Diluted extract 1:16	880	830	780	1,000
Original extract	820	110	3	0
Original extract 1: 2	800	30	3	0
Original extract 1: 4	700	40	1	0
Original extract 1: 8	890	610	330	24
Original extract 1:16	900	800	600	340

With this knowledge the experiment was repeated, the method being so modified as to avoid dilution. Instead of dissolving the precipitate in distilled water it was transferred to the dialyzing sac in the solid condition and allowed to go into solution in the small amount of water that entered the sac as a result of the differences in osmotic pressure. In this way it was found possible to dialyze the precipitate free from ammonium sulphate without allowing the volume at any time to increase above the initial volume from which the precipitate was obtained.

The products isolated by this method still show variations, but the results are sufficiently constant to warrant the provisional con-

clusion that the bactericidal substance is probably precipitated quantitatively by full saturation with ammonium sulphate (table V).

TABLE V.

Precipitation with Ammonium Sulphate.

To a sample of the salt-free extract pulverized ammonium sulphate was added to saturation, and the mixture was allowed to stand for one hour at room temperature. The resulting precipitate was thrown down by centrifugalization, washed once with a small amount of standard ammonium sulphate, and pressed between sheets of sterile filter paper to remove as much of the ammonium sulphate solution as possible. The solid precipitate was now transferred to a celloidin sac and dialyzed five times at 4° C. against 1,500 c.c. of double distilled water.

During the dialysis the precipitate went into solution in the small amount of water entering the sac, this volume increasing by the end of the dialysis to about two thirds of the original volume of the extract from which the precipitate was obtained. At the end of the dialysis this volume was made up to the original volume by the addition of distilled water, and the bactericidal power of the resulting solution was compared with that of the original extract. Test organism, *B. typhosus*.

Material tested.	Time of plating.			
	1 min.	2 hrs.	5 hrs.	24 hrs.
Precipitate	580	40	4	0
Precipitate 1: 2	580	20	3	0
Precipitate 1: 4	470	50	2	0
Precipitate 1: 8	1,020	1,120	720	20
Precipitate 1:16	1,010	940	810	550
Original extract	920	60	8	0
Original extract 1: 2	940	20	3	0
Original extract 1: 4	900	30	1	0
Original extract 1: 8	970	350	160	110
Original extract 1:16	1,040	870	770	810

The existence of antibactericidal substances in the extract not precipitated by ammonium sulphate may necessitate a subsequent modification of this conclusion. A study of precipitation by partial saturation with ammonium sulphate and precipitation with other salts is in progress, and will be reported in a later paper.

The earlier attempts to determine the relation of the bactericidal substance to alcoholic precipitation were also confusing. Samples of the extract were added to alcohols of various concentrations, the

resulting precipitates were thrown down by centrifugalization, and the supernatant alcohol decanted and evaporated to dryness. By this means the leucocytic extract was separated into two fractions,—an alcohol-soluble and an alcohol-insoluble part. These two fractions were taken up, either in distilled water or in physiological saline, or usually in some dilution of physiological saline, as fifth-physiological or tenth-physiological, and tested for their bactericidal properties.

In estimating bactericidal power it is desirable to have the osmotic pressures the same in the various parallel fluids to be tested. The dissolving and diluting fluids used were, therefore, those that would make the osmotic pressures equal throughout the determination. The results of these determinations were contradictory and uncertain.

Part of this early confusion I can now attribute to a destruction of the bacteriolysin as a result of dilution, a source of error not suspected at the time the determinations were made. A second source of error was subsequently discovered, the wholly unsuspected antibacteriolytic action of sodium chlorid.

It was early observed that the extracts from horse leucocytes usually increased considerably in bactericidal power on dialysis (table VI). This was at first attributed to the removal of inhibiting proteids as a result of the routine reheating at the end of dialysis, but a subsequent study showed that dialysis alone caused such an increase.

TABLE VI.

Effect of Dialysis on Bacteriolysin.

Samples of extract from horse leucocytes tested before and after dialysis. The dialyzed sample was reheated to insure sterility. Test organism, *B. typhosus*.

	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Before dialysis	780	560	330	900
After dialysis	550	180	8	0

Sodium chlorid added to a salt-free extract generally decreases its bactericidal power (table VII), the presence of 0.85 per cent.

sodium chlorid often being sufficient to inhibit completely bacteriolysis, while as little as 0.2 per cent. sodium chlorid produces an appreciable lessening. There was, therefore, sufficient sodium chlorid in most of the solvents employed in the earlier tests to render these determinations inconclusive. In repeating the work, distilled water was selected as the dissolving and diluting medium.

TABLE VII.

Effect of Sodium Chlorid on Bacteriolysin.

Samples of a salt-free purified bacteriolysin from horse leucocytes tested for its bactericidal power both alone and in the presence of increasing amounts of sodium chlorid. Test organism, *B. typhosus.*

Material tested.	Time of plating.			
	1 min.	1½ hrs.	4½ hrs.	24 hrs.
Bacteriolysin, water.....	790	210	15	0
Bacteriolysin, +0.2 per cent. sodium chlorid.....	700	120	25	2,500
Bacteriolysin, +0.4 per cent. sodium chlorid.....	1,090	480	90	5,000
Bacteriolysin, +0.85 per cent. sodium chlorid.....	1,180	750	660	10,000

If leucocytic extract is added to absolute alcohol, a precipitate forms immediately. If this precipitate is washed with absolute ether and dried *in vacuo,* there is obtained a product soluble in distilled water, possessing about half the bactericidal power of the original extract. If, instead of immediately washing the precipitate with ether, it is washed with absolute alcohol, and is then dried, the final product is largely insoluble. By employing distilled water, however, a fairly strong bactericidal agent can be extracted from this precipitate. This water extract is free from hemoglobin and is presumably free from many other contaminating proteids. It represents the bactericidal agent in the highest degree of purity thus far obtained. The properties of this purified bacteriolysin are now under investigation.

No final conclusion can yet be drawn as to the nature of the bactericidal substance in leucocytic extract. Its behavior toward ammonium sulphate and alcohol are sufficient, however, to warrant the belief that the bactericidal substance is possibly an enzyme. Upon this supposition the investigation is being continued.

CONCLUSIONS.

1. The bactericidal agent extracted from horse leucocytes is apparently precipitated quantitatively by full saturation with ammonium sulphate.

2. The bactericidal agent is apparently precipitated by absolute alcohol, and is not rendered insoluble by a short contact with alcohol. The agent resembled in this feature certain enzymes which can be isolated and purified by alcoholic precipitation.