STUDIES ON A BACTERICIDAL AGENT EXTRACTED FROM A SOIL BACILLUS

III. Preparation and Activity of a Protein-Free Fraction

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In previous reports the preparation and properties of an agent extracted from cultures of an unidentified soil bacillus were described; this agent exerts a marked bactericidal effect on Gram-positive microorganisms.[1,2,3] When prepared from culture autolysates according to the method outlined in an earlier publication, the bactericidal agent is found to be associated with a protein precipitable at pH 4.5; evidence has already been presented, however, indicating that the active material itself is not a protein.[2] It is shown in the present paper that the bactericidal activity of the original preparation was due to a fraction which is soluble in alcohol, acetone, and dioxane, but insoluble in water, and which is entirely free of protein.

EXPERIMENTAL

Separation of the Bactericidal Agent from the Protein Fraction.—A solution of the bactericidal agent associated with the protein fraction precipitable at pH 4.5, was prepared by the method previously described.[2] When a given amount of this active solution is added to 5 volumes of acid acetone (containing for instance 1 cc. concentrated HCl per liter of acetone), a whitish precipitate immediately forms, while the acetone assumes a yellow color. The precipitate consists of a protein which is soluble in water, precipitable at pH 4.5, and soluble again in an excess of acid.

When the acetone is removed from the acetone-soluble fraction by distillation at reduced pressure, there remains in the distillation flask a brown material insoluble in water, but very soluble in acetone and alcohol.

Both the protein fraction (water-soluble) and the acetone-soluble fraction (water-insoluble) have been tested, in comparison with the original bacteri-

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[1] Dubos, R. J., *Proc. Soc. Exp. Biol. and Med.*, 1939, **40**, 311.

[2] Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 1.

[3] Dubos, R. J., *J. Exp. Med.*, 1939, **70**, 11.

cidal extract, to determine their respective ability to inactivate the glucose dehydrogenase of Gram-positive microorganisms, to cause the lysis of pneumococci and staphylococci, and to protect mice against infection with virulent pneumococci and streptococci. The protein fraction has been found to be completely inactive in all these tests, whereas the acetone-soluble, water-insoluble fraction retains, both qualitatively and quantitatively, the activity of the original material.

Solubility Properties of the Active Fraction.—The active material can be conveniently precipitated from an acetone solution by adding the latter to 10 volumes of water containing electrolytes (0.5 per cent NaCl for instance). The flocculent precipitate which forms under these conditions can be recovered by filtration or centrifugation and contains all the bactericidal activity of the original acetone solution.

The active material is insoluble in chloroform, sulfuric ether, petroleum ether, benzol, and toluol, as well as in water. On the contrary, it is very soluble, and remains active, in acetone, alcohol, dioxane, pyridine, and glacial acetic acid.

When alcoholic or acetone solutions are diluted with large volumes of distilled water, stable aqueous solutions (or suspensions) are obtained; the active material, however, precipitates immediately and completely upon the addition of salts. Stable aqueous solutions are also obtained when alcoholic solutions are diluted with large volumes of 5 per cent glucose in distilled water. This property permits the preparation of isotonic solutions for injection into experimental animals.

Preparation of Lot 2 of the Bactericidal Agent.—To date, 13 different lots of the bactericidal agent have been obtained in an active form, free of protein. We shall describe in some details the preparation of lot 2, since the latter has been used extensively in a number of experiments to be reported in this and other publications.

80 liters of 1 per cent tryptone broth were inoculated and incubated under the conditions previously described.[2] After 4 days incubation at 37°C., the whole autolyzed culture was adjusted to pH 4.5 by the addition of 210 cc. of concentrated HCl. A heavy precipitate formed containing the cellular material and the active bactericidal substance. The precipitate was separated by filtration through filter paper, and the clear filtrate was discarded.

The precipitate was taken up in 5 liters of acetone containing 5 cc. of concentrated HCl and was allowed to stand for 24 hours in this acid medium. The acetone-soluble fraction was separated by filtration, and most of the acetone was removed by distillation under reduced pressure. The residue left in the distillation flask was thrown into 5 liters of water to which had been added 150 cc. of concentrated HCl. A heavy pre-

cipitate formed; this was filtered through a Büchner funnel, the filtrate discarded, and the precipitate desiccated *in vacuo* over P_2O_5; the dry material weighed 15 gm.

The dry material was extracted repeatedly with sulfuric ether and then taken up in 300 cc. of 95 per cent alcohol, in which a small fraction remained insoluble; the insoluble fraction was discarded and the alcohol-soluble material was precipitated by adding it to 1.5 liters of water containing 30 cc. of concentrated HCl. The precipitate, recovered by filtration through a Büchner funnel, was desiccated *in vacuo*. The dry precipitate was taken up in 300 cc. of dioxane in which a fraction remained insoluble; the insoluble fraction was discarded; the soluble material was again precipitated in 1.5 liters of water containing 30 cc. of concentrated HCl. The precipitate, desiccated *in vacuo* over P_2O_5, weighed 8 gm. and was designated lot 2.

Lot 2 appears as a greyish powder which is readily and completely soluble in acetone, alcohol, and dioxane to the extent of at least 100 mg. per cc. at room temperature. From these concentrated solutions one can obtain clear and stable dilutions, containing up to 1 mg. per cc., in distilled water or in 5 per cent glucose solution; the active material, however, immediately precipitates out of the aqueous medium upon the addition of salt.

An alcoholic solution, containing 20 mg. per cc., has now been kept in the laboratory at room temperature for 3 months and, as far as can be detected, has retained unimpaired its bactericidal activity when tested *in vitro* or *in vivo*.

Although the dry material of lot 2 contains 12.5 per cent nitrogen, it does not give any of the common protein tests (biuret, xanthoproteic, Millon, Hopkins-Cole, Sakaguchi), even when used in high concentrations. The material also fails to give any of the tests for carbohydrates, fats, or sterols.

Activity of the Protein-Free Fraction in Vitro.—The protein-free preparations of the bactericidal agent are effective *in vitro* against all the Gram-positive microorganisms so far tested; they inhibit their growth in laboratory media and destroy their glucose dehydrogenase; they also cause the lysis of pneumococci, staphylococci, and of the cells of an unidentified Gram-positive rod. In short, all the tests of bactericidal activity described in an earlier publication[2] have been duplicated with the new protein-free fractions; a few examples will be given to illustrate the activity of lot 2 *in vitro*.

Pneumococcus (Type I), *Streptococcus hemolyticus* (group A type 6), and *Staphylococcus aureus* were grown in beef heart infusion peptone broth for 6 hours. The grown cultures were adjusted to pH 7.4 by the addition of M/1 K_2HPO_4 and distributed in 5 cc. amounts into test tubes. Different amounts of lot 2, diluted to 1 cc. in distilled water, were added as indicated in Table I and the mixtures were incubated for 4 hours

at 37°C. The bactericidal effect of the various dilutions of the active substance was then established by the following tests: (*a*) viability of the cells was determined by streaking the mixtures on blood agar plates; (*b*) inhibition of the glucose dehydrogenase was determined by adding methylene blue (1 cc. of 0.001 M solution) and glucose (1 cc. of 10 per cent solution) to the mixtures of cell suspension and bactericidal agent, then sealing with vaseline and observing reduction of the dye after 2 hours incubation at 37°C.; (*c*) lysis of the cells was recognized by decrease in turbidity of the bacterial suspensions, and was confirmed by microscopic examination.

TABLE I

Effect of a Protein-Free Preparation of the Bactericidal Substance on Different Bacterial Species in Vitro

Test organism		Amount of bactericidal substance (mg. per cc. of culture)						
		0.040	0.020	0.010	0.004	0.002	0.001	0
Diplococcus pneumoniae Type I	Viability*	−	−	−	−	−	+	++++
	Reductase†	NR	NR	NR	NR	NR	NR	CR
	Lysis‡	C	C	C	C	C	P	N
Streptococcus hemolyticus group A type 6	Viability	−	−	−	−	−	+++	++++
	Reductase	NR	NR	NR	NR	NR	PR	CR
	Lysis	N	N	N	N	N	N	N
Staphylococcus aureus	Viability	−	−	−	++++	++++	++++	++++
	Reductase	NR	NR	NR	CR	CR	CR	CR
	Lysis	C	C	N	N	N	N	N

* − = no growth on blood agar. + = much reduced growth on blood agar. ++++ = abundant growth on blood agar.

† NR = no reduction of methylene blue. PR = partial reduction of methylene blue. CR = complete reduction of methylene blue.

‡ N = no lysis. P = partial lysis. C = complete lysis.

The results presented in Table I show that 0.002 mg. of the protein-free fraction is sufficient to sterilize 1 cc. of broth culture of Pneumococcus (Type I) or of hemolytic streptococcus (group A type 6) in 4 hours at 37°C.; sterilization of 1 cc. of staphylococcus culture requires 0.010 mg. of the same material. Although death of the cells was accompanied by inactivation of their glucose dehydrogenase in every instance, bacteriolysis occurred only in the case of pneumococci and staphylococci; it has been pointed out in an earlier publication[2] that the lysis caused by the bactericidal agent is only a secondary process, which follows some other primary injury inflicted upon the bacterial cell.

The bactericidal effect of the protein-free fraction also expresses itself in

an inhibition of the growth of the susceptible species in laboratory media, as is shown in the following experiment.

100 cc. of beef heart infusion peptone broth were inoculated with 1 cc. of pneumococcus culture. The inoculated medium was distributed in 5 cc. amounts into 20 test tubes; 9 of these tubes received varying amounts of lot 2 which had been heated in a boiling water bath for 10 minutes prior to being added to the inoculated medium; 9 other tubes received the same amounts of the original unheated preparation. 2 tubes of the same inoculated medium received no bactericidal agent and served as controls. The different mixtures were incubated at 37°C. and observed for the development of growth of pneumococci.

TABLE II

Inhibition of the Growth of Pneumococci by the Protein-Free Fraction of the Bactericidal Agent

Inoculum Pneumococcus Type I	Amount of agent per 5 cc. of broth	Time required for growth to develop in the presence of	
		Bactericidal agent unheated	Bactericidal agent heated at 100°C.
cc.	*mg.*	*hrs.*	*hrs.*
0.05	0.001	—*	—
"	0.0003	—	—
"	0.0001	—	—
"	0.000,03	—	—
"	0.000,01	48	48
"	0.000,003	48	48
"	0.000,001	24	24
"	0.000,000,3	24	24
"	0.000,000,1	18	18
"	0	12	12

* — = no growth.

As appears from the results presented in Table II, 0.000,003 mg. of the protein-free fraction added to 5 cc. of broth was sufficient to retard considerably the growth of pneumococci (inoculum 0.05 cc.); no growth developed when the amount of bactericidal agent was increased to 0.000,03 mg. It is also remarkable that the bactericidal agent retains its activity *in vitro* after having been heated for 10 minutes at boiling temperature. It must be stated here, however, that the same heated preparations, which are still fully active *in vitro*, have lost most of their *in vivo* activity. This discrepancy accounts for apparently conflicting statements in two earlier publications. In one of them,[1] the bactericidal substance was reported to be heat-labile; this was claimed on the basis of protection tests in mice. In another publication, the material was said to resist boiling at pH 2.0 and

pH 9.0; this statement was based on the results of *in vitro* tests. The nature of the phenomenon which determines the different behavior *in vitro* and *in vivo* is being investigated at the present time.

Similar tests have been carried out with a number of different species of microorganisms. In general it can be said that all the Gram-positive species so far tested have been found susceptible to the bactericidal action of the protein-free fraction; on the contrary, the same material is ineffective against Gram-negative bacilli; Gram-negative cocci and acid-fast bacilli have not yet been tested.

TABLE III

Protective Effect of a Single Dose of the Protein-Free Fraction

Infective dose of Pneumococcus Type I	Treatment	Number of mice	Results					
cc.	*mg.*							
0.0001	0.020	6	S	S	S	S	S	S
"	0.010	6	S	S	S	S	S	S
"	0.005	6	D 96	S	S	S	S	S
"	0.002	6	D 48	D 72	D 72	D 72	S	S
"	0.001	6	D 24	D 48	D 48	D 48	D 48	D 72
"	0	3	D 24		D 48		D 48	
0.000,001	0	1	D 48					
0.000,000,1	0	1	D 48					
0.000,000,01	0	1	D 48					

S = survival (6 days observation period).

D = death (numeral indicates the number of hours after infection).

Protective Action of the Protein-Free Fraction in Vivo.—The protective activity of the different fractions obtained during the present work has been established by determining the minimal amount of bactericidal agent injected in a single dose which would protect mice against 0.0001 cc. of Type I pneumococcus culture of maximal virulence (*i.e.*, 10,000 fatal doses). The following experiment illustrates the activity of lot 2. The cultures and techniques used in the protection experiments were in all respects the same as those described in an earlier publication.[3]

Mice were infected by the intraabdominal route with 0.0001 cc. of culture of virulent pneumococci Type I; they were subsequently treated, within a few minutes after injection of the infective dose, with varying amounts of lot 2 also injected intraabdominally.

It is clear from the results presented in Table III that mice can be protected against 10,000 fatal doses of Pneumococcus Type I with a single dose of 0.005 mg. of the protein-free fraction of the bactericidal agent. As

will appear from the following experiment, it is also possible to protect mice against infection with larger amounts of pneumococcus cultures by the use of repeated treatments with the bactericidal agent.

Mice were inoculated with varying amounts of cultures of virulent pneumococci of Types I and III. They were treated on 3 consecutive days; 0.020 mg. of lot 2 was given within a few minutes after injection of the infective inoculum, 0.010 mg. 24 hours later and again 0.010 mg. 48 hours later.

TABLE IV

Protective Effect of Repeated Treatments with the Protein-Free Fraction

Infective dose of Pneumococcus	Treatment on 3 consecutive days			Number of mice	Results					
cc.	*mg.*	*mg.*	*mg.*							
Type I 0.1	0.020	0.010	0.010	6	D 48	S	S	S	S	S
" 0.01	"	"	"	6	D 72	S	S	S	S	S
" 0.001	"	"	"	6	D 48	D 48	S	S	S	S
" 0.0001	"	"	"	6	S	S	S	S	S	S
" 0.000,001	0	0	0	1	D 44					
" 0.000,000,1	0	0	0	1	D 46					
" 0.000,000,01	0	0	0	1	D 72					
Type III 0.1	0.020	0.010	0.010	6	D 24	D 24	D 48	D 72	D 72	S
" 0.01	"	"	"	6	D 72	D 72	D 86	S	S	S
" 0.001	"	"	"	6	D 72	S	S	S	S	S
" 0.0001	"	"	"	6	S	S	S	S	S	S
" 0.000,001	0	0	0	1	D 44					
" 0.000,000,1	0	0	0	1	D 44					
" 0.000,000,01	0	0	0	1	D 44					

The results presented in Table IV demonstrate the marked protective effect of the protein-free fraction of the bactericidal agent against experimental infection of mice with pneumococci of Types I and III. Similar results have been obtained with other types of pneumococci, and it has also been found that small amounts of the same material not only protect mice against infection with virulent pneumococci but that they also exert a curative effect against an established infection, when treatment is first instituted several hours after injection of the infective inoculum. In other words, all the protective and curative results obtained with the preparations of bactericidal agent described in the first two papers of this series have been duplicated with the new, protein-free preparations. Finally, it may be stated that experiments carried out by Dr. R. C. Lancefield have

established that the same material is also capable of protecting mice against infection with virulent hemolytic streptococci of group A and group C.

DISCUSSION

The preparation of the bactericidal agent described in earlier publications[1-3] contained a protein precipitable at pH 4.5. The purified preparation used in the present experiments is soluble and stable in alcohol, acetone, dioxane, pyridine, and glacial acetic acid, but insoluble in water, chloroform, sulfuric ether, petrolic ether, benzol, and toluol. Although the new preparation contains 12.5 per cent nitrogen, it entirely fails to give any of the protein tests. Qualitatively, it exhibits all the bactericidal properties, both *in vitro* and *in vivo*, which have been described for the protein-containing preparations. Quantitatively the new purified, protein-free preparation, is about 100 times more active than the earlier material. It appears very likely, therefore, that the active substance is not a protein.

SUMMARY

A cell-free extract of cultures of an unidentified soil bacillus, which exerts a bactericidal effect on Gram-positive microorganisms, has been described in previous reports; the first active preparations which were obtained were found to contain a protein precipitable at pH 4.5. It is shown in the present report that the bactericidal agent can be obtained in an active form free of protein. The new purified preparations retain all the activity of the original material, both *in vitro* and *in vivo*.