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A Bacteriophage Active Against a Virulent Hemolytic Streptococcus.

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We reported[1] last year the finding of a bacteriophage against a virulent hemolytic streptococcus. Such a lytic principle active against streptococcus has been reported so rarely, if at all, that it seems desirable to state our results in some detail.

The organism against which the phage was originally active was isolated about 3 years ago from a spontaneous fatal infection in a stock rabbit. At autopsy, the animal showed marked thoracic lesions, myocarditis, pericarditis, bronchopneumonia, fibrino-purulent pleurisy, extensive empyema with relatively little grossly noticeable below the diaphragm save an acute splenic tumor. The organism was readily obtained in pure culture from these lesions and also from the heart's blood. It grows on sheep blood agar as a very moist, translucent, mucoid colony, somewhat similar to that of *Pneumococcus mucosus,* but it produces a wide zone of hemolysis on the plate. One cc. of a 12 hour broth culture will cause complete hemolysis of a 5 per cent suspension of washed sheep red blood cells when incubated for one hour at 37° C. It does not ferment inulin and is bile insoluble, so we consider it an example of a *Streptococcus mucosus.* It closely resembles the organisms found in cases of septic sore throat. The various fermentation reactions are recorded in Table I.

The organism is Gram positive, but is more readily decolorized than many streptococci, and, frequently, partially decolorized cocci may be observed in a chain of Gram positive organisms. It appears in chains of moderate length, the individual members of the chain usually being spherical and variable in size. Capsules can be readily demonstrated by any of the commonly used methods.

This organism proved virulent for other rabbits, causing a fatal septicemia in 5 to 7 days, when injected intravenously into full grown animals in amounts of .001 cc. of a broth culture. It has, over a period of three years, shown a strong tendency to localize in the thoracic organs, especially the heart, and has maintained its original virulence to a marked degree for many months without animal passage.

The common finding of the lytic principle in sewage led us to

TABLE I.
Fermentation Reactions.

	Dextrose	Mannitol	Maltose	Xylose	Lactose	Salicin	Sucrose	Raffinose	Dulcite	Inulin	Dextrin
Streptococcus mucosus (Rabbit) 48 hours	+	|	+	|	+	+	+	|	|	|	+
Streptococcus mucosus (Guinea pig) 48 hours	+	|	+	|	+	+	+	|	|	|	+

wonder whether the activated sludge process, as used in Milwaukee, Wis., with its unusually high content of aerobic spore formers, might not be a source of phage against other organisms than the common members of the colon (dysentery) typhosus group. This possibility seemed especially interesting because of the finding by Nicolle[2] that a filtrate of *B. subtilis* would dissolve pneumococci. Accordingly, a dozen different streptococci and pneumococci and half as many members of the colon typhosus group were grown in flasks containing 50 cc. beef infusion broth plus 5 cc. of the activated sludge. After 3 to 4 days' incubation this mixture was centrifuged, filtered through a Mandler filter, and tested for the presence of phage against the organism employed. If the result was negative, the filtrate was reinoculated with the organism and the activated sludge and the whole process repeated at least ten times. We very

promptly obtained a lytic principle active against the different members of the colon typhosus group used, and on the second cultivation attempt we succeeded in obtaining the phage active against the *Streptococcus mucosus*. All efforts to obtain a lytic principle active against other streptococci and pneumococci failed.

This bacteriophage causes the transmissible lysis of this particular streptococcus, and in other ways acts in a typical manner. We have commonly employed standard beef infusion broth with a pH of 7.6 and the organisms from a broth culture from 5 to 24 hours old. The phage made up in increasing dilutions in this broth will inhibit the growth of the specific organism, *Streptococcus mucosus*, in dilutions usually as high as 1×10^{-9}, and occasionally as high as 1×10^{-13} when two drops of a turbid young broth culture are added to the phage dilutions and the whole incubated for 24 hours. Quantitative subculture platings on blood agar show that most of the tubes are not actually sterile, although the number of living organisms is small. We have obtained actual sterility only by using relatively large amounts of the phage, 10 per cent or 33⅓ per cent of the total volume of the broth. Not always have these concentrations given sterility. These concentrations have been more effective in giving complete sterilization than has 100 per cent phage.

On blood agar tubes or plates the phage causes a marked diminution in the number of colonies planted on a given area. Only rarely, however, does one observe the moth eaten type of colony so common with the phagic action on members of the colon typhosus group. This is due partly, we believe, to the small size of the colonies of this organism. When a drop or two of this phage is added to a tube of young broth culture of the organism, complete clearing of the tube takes place when incubated for a few hours.

We succeeded in obtaining a similar phage against this organism by the use of 2 other samples of the Milwaukee activated sludge, although once it took 6 cultivations to obtain this result, the filtrate from the fifth cultivation showing no phage action. We also succeeded in obtaining the phage upon the first cultivation with the effluent from Madison sewage, which is always a ready source of phage active against the various members of the colon typhoid group.

With the filtered products of its own metabolism, repeated cultivation of this streptococcus failed to produce any phagic action, even when recultivation was continued thirteen times.

Our notion that the aerobic spore formers of the activated sludge might be responsible in initiating the production of the lytic agent seems to be without foundation. We tried repeated serial cultiva-

tions of the streptococcus with a sterile filtrate of the proteases and digestion products from *B. subtilis* and failed to obtain any phagic action.

We endeavored to adapt our phage to 6 other hemolytic streptococci (2 of them isolated by G. Howard Brown from cases of septic sore throat) and to each of the 3 fixed types of pneumococci by all of the usual methods, and by various modifications of our own, but entirely without success.

The fact that our organism was obtained originally from a spontaneous infection in a rabbit, and is definitely virulent for that animal, has given us a favorable opportunity to try the effect of the phage in the treatment of a disease in its original host. A number of series of animals were, therefore, treated with the lytic principal. In 3 series, treatment was begun before the organisms were injected, and in 3 series, immediately after the injection of the organisms. Varying dosages of the phage were employed up to 20 cc. daily and various routes were used (*per os,* intravenous and intraperitoneal), but in no case did the treated animals do better than the untreated. In fact, in all instances in which large amounts of the phage were used, the treated animals died before the control animals, and showed the same type of lesions and a similar degree of severity.

We found no evidence of the passage of the phage into the blood stream when as much as 10 cc. was injected *per os* or intraperitoneally, nor did the phage remain in the blood stream any considerable length of time when injected directly into the vein. Tests of blood serum taken 2 hours and also 2 days after such treatments showed no bacteriophagic action. This elimination of the phage from the blood stream is due, at least partly, to adsorption of the phage directly by the blood cells. Experiments both with defibrinated sheep, and rabbit blood, plus the phage, incubated and shaken together for 2 hours, showed marked, sometimes complete, inhibition of the phage.

One of the obvious difficulties in any possible use of the phage as a therapeutic agent lies in the fact that the phagic broth must also contain various toxic elements from the metabolism and cleavage of the organisms. The separation, therefore, of the phage from these toxic products would seem to be a necessary step. We have tried both the acetone and the alcoholic precipitation methods described by Kabeshima,[3] but our phage was completely destroyed by the alcohol method, and markedly reduced in potency by the acetone method.

Various adsorption reagents were employed: ordinary kaolin;

Lloyd's reagent, and charcoals. The best adsorbing agent proved to be Rankanite A, an activated charcoal perfected by the Chemical Warfare Service during the war. (We obtained this through the courtesy of Professor Victor Lenher of the University of Wisconsin.) Twenty grams of this charcoal, combined with 100 cc. of the broth phage, shaken for one hour in a machine, and refrigerated over night, will completely adsorb the phage from the broth. Tests of the supernatant show no evidence of phage even when the broth had originally a titer of one to a billion.

After striving without success for the better part of a year to obtain a lytic principle active against some other streptococcus or pneumococcus either directly or by adaptation of this phage, we accidentally obtained an organism very like our original *Streptococcus mucosus* which proved to be susceptible to our original phage. A stock guinea pig, which had been recently received from the country, died. At autopsy, which showed general thoracic involvement, we recovered from the pleural exudate, the lungs and the heart's blood a mucoid hemolytic streptococcus which was microscopically and culturally similar to our original organism. It also gave the same fermentation reactions.

This organism was readily lysed by the *Streptococcus mucosus* phage, but only when higher concentrations were used than were effective against the original organism. For example, the first titration made showed effective lysis of the guinea pig organism in a dilution of phage as high as 1 to 10,000 while the same phage was active against the rabbit organism in a concentration of 1 to 10,000,000. Subsequently we successfully obtained a phage against the guinea pig strain by cultivating it together with Madison sewage effluent. This phage was equally active against the original rabbit strain. We made no attempt to determine the antigenic identity of the two strains by the use of immune reactions but from all other points of view they were closely alike.

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[1] Clark, P. F., and Clark, A. S., *J. Bact.*, 1926, xi, 89.

[2] Nicolle, *Ann. de l'Inst. Pasteur*, 1907, xxi, 613.

[3] Kabeshima, *Compt. rend. Soc. de Biol.*, 1920, lxxxiii, 471.