Bacteriology. — *"On the Bacteriophage and the Self-purification of Water"*, by Prof. P. C. FLU.

(Communicated at the meeting of Dec. 30, 1922).

In 1896 HANKIN [1]) reported that the water of various rivers in India, i. a. the Yumna and the Ganges possesses the property of rapidly destroying cholera-vibriones. He was disposed to ascribe this property to a volatile substance, which he assumed to occur in the water of the said rivers.

Subsequent experimenters have demonstrated that all so-called surface-waters have the faculty of exterminating microbes, notably fortuitous pathogenic germs, at a rate depending on the nature of the water and the temperature of the environment.

EMMERICH, who studied this phenomenon, the so-called selfpurification of water, believed that in this process the part of germicide must be assigned to protozoa (Rhizopods, Flagellates and Ciliates) which occur in every surface-water. This view was adhered to by nearly all inquirers, who had occupied themselves with the phenomenon.

D'HÉRELLE refers in his work "Le bactériophage, son rôle dans l'immunité" to the phenomenon observed by HANKIN which he thoroughly believes to be merely the effect of a bacteriophage present in the water.

Now, we know that bacteriophages are inactivated at a temperature above 75° C., and that HANKIN could heat water of the said rivers in a closed vessel (a sealed-up glass tube) for half an hour up to 115° C, without depriving it of its bactericidal capacity. We also know that, on heating up the Yumna, and the Ganges-water during the same interval and up to the same temperature (but in an open vessel), it really lost its bactericidal capacity.

Now, in view of these facts it will be difficult to side with D'HÉRELLE, although we must admit at the same time that protozoal action does not explain the phenomenon any better.

Still, it cannot be denied that after D'HÉRELLE's significant discovery and after the establishment of the presence of bacteriophages attacking various germs in all sorts of surface-waters, in seawater and even in the effluent from septic-tanks and from oxidation-beds,

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[1]) Annales de l'Instituut PASTEUR Vol. X pag. 175 and 511.

an interpretation of the self-purification of water can hardly be afforded without reckoning with the bacteriophage.

If a special inquiry in this direction were to show that bacteriophages play a more prominent part in the process of self-purification than has hitherto been assumed, we should not only have to revise and modify our conceptions of and our insight into this self-purification of water and our views concerning the action of sand-filters and oxidation-beds, but also a broad field would be opened up for studying the biological cleansing of sewage.

Like many others I also became convinced by my experimentation in India of the prominent part played by protozoa in the destruction of micro-organisms in the surface-water.

For this reason I deemed it a matter of importance to ascertain:

a. whether in surface-water, e. g. that in and about Leyden, bacteriophage could be found, and whether the self-purification of that water was in any way due to bacteriophages that might occur in it.

b. whether in surface-water, polluted intentionally with a profusion of pathogenic micro-organisms, and allowed, to purify itself, bacteriophages are to be observed that may have annihilated the germs.

c. the influence which is played on the purification by substances that kill the protozoa but do not injure the bacteriophages.

d. whether protozoa and bacteriophages combined may accelerate the process of self-purification.

To this end the following experiments were performed:

On the 2d of June 100 c.c. of various samples of Leyden water were mixed every time with a concentrated broth. The mixture stood during 24 hours at 37° C. and was then filtered first through rock-meal and subsequently through a "bougie". The filtrate was mixed in quantities of 0,5; 0,2; 0,1; and 0,05 c.c. with broth, which was afterwards inoculated with an 18-hour-old Flexner-culture. For an examination for bacteriophage a smear-culture was made on agartubes of the broth thus prepared. After an incubation of 24 hours at 37° C. an estimination was made for "phages".

The result is that from the examined waters bacteriophages can be isolated that react especially to Flexner but also have an action on other intestinal bacteria.

Thus the isolated bacteriophages annihilate all the Flexner, Y, and Shiga Kruse stocks of our collection.

They also have an action on bacillus faecalis alcaligenes, on a proteus and a proteus X 19, but do not act upon Typhus, Paratyphus A. and B. or Enteridite Gärtner, neither on two coli-stocks of our collection.

Neither was any effect of the bacteriophages on cholera-vibriones at all apparent.

This result could be expected, as it is known that from the dejecta of fowls and horses a nearly always highly active bacteriophage antibacteria dysenteriae can be isolated and the surface-water in and about Leyden is being constantly polluted on a large scale by the excrements of a number of living beings, also by those of horses and fowls.

Anyhow this inquiry teaches us that bacteriophage occurs in the surface-water of Leyden.

On the 2d of June quantities of 5 Liters of various kinds of Leydenwater were infected every time with two loopfuls of a 24-hour-old cholera-culture. The infected water was placed in large glass receptacles in diffuse daylight at room temperature (15° C.).

On the 21st of June we examined two quanta of 25 c.c. of water; in neither of those samples could cholera-vibriones be detected.

Of every sample of 5 L. 25 c.c. was examined for bacteriophages by mixing the water with $^1/_{10}$ of the volume of concentrated broth, and inoculating the mixture with a loopful of an 18-hour-old cholera-culture.

After an incubation of 24 hours at 37° C. the sample was examined in the usual way for bacteriophage anticholera-vibriones. The result was negative.

On the 24th of June three flasks were filled each with 0,5 L. of Rijnwater, in which, as our examination had proved, bacteriophage antibacteria dysenteriae was present.

Flask I was inoculated with the whole cholera-culture of a sloped agar tube; flask II in the same manner with typhus-bacilli; and flask III with Shiga-Kruse bacilli.

The fluid of each of the three flasks became very turbid and was placed at room-temperature in diffuse daylight.

On the fifth of July the fluid of each of the three flasks became lucid and was examined for bacteriophage in the ordinary way. In all the flasks we found bacteriophage antidysenteriae, which was present in the water already before the beginning of the experiment, but in the typhus-flask not any bacteriophage antityphus was found, no more than bacteriophage anticholera in the cholera-flask.

The flask infected with Shiga did not become lucid sooner than the one infected with typhus and cholera, which might have been

expected if a protozoal action had been assisted by the bacteriophage antidysenteriae present in the water.

In each flask the number of protozoa increased already two days after the inoculation with the mass of bacteria. Their number was greatest one day before the contents of the flasks became lucid, whereas it decreased after the clarification had been completed; some of them were transformed into cysts.

Again a culture, equal to the one at the beginning of the experiment was transplanted into the flasks in which the typhus-bacteria and the cholera-vibriones had disappeared. The same was repeated twice when, after about ten days the contents had clarified again.

After each new infection the number of protozoa was augmented, as with the first, reached its maximum shortly before the clarification and decreased again after it. Every time a portion of the protozoa were seen to turn into cysts.

When the contents of the flasks had become quite clear again after the fourth infection, another examination was performed for bacteriophage antityphus abdominalis and anticholera vibriones. The result was absolutely negative.

So these experiments go to show that large crowds of typhus-bacteria and cholera-vibriones may disappear without any interference whatever of bacteriophages, from water into which they were introduced fortuitously or intentionally. Even in water containing a bacteriophage anti-bacteria-dysenteriae the B. dysenteriae do not disappear quicker than other bacteria not attacked by bacteriophage.

It was nevertheless of interest to examine especially the influence of the presence or the absence of bacteriophage anti-shiga on the rate of disappearance of B. dysenteriae from the water.

Two series of experiments were accordingly carried out.

In the first series the fate of B. dysenteriae in unfiltered water was compared with that of the same bacilli in filtered water.

Protozoa cannot pass through a filter impervious to bacteria, whereas the bacteriophage is let through.

In the second series a comparison was made of the rapidity of the selfpurification process of bacteriophage containing water that was or was not mixed wit KCN.

The results of these tests, which were every time the same, are reported below.

Vlietwater, which contains bacteriophage, was used for the inquiry. Part of it was filtered through a Berkefeld-filter. A control-experiment showed that this water is free from bacteria and protozoa.

Part of the filtered, as well as the unfiltered water was infected

with another quantity of highly active bacteriophage (0,2 cc. to 10 cc. of liquid. The bacteriophage was still active in a dilution of 10^{-10}). Bacteriophage was superadded to demonstrate its influence still more conclusively than could be done with the bacteriophage already occurring in the Vlietwater.

The subjoined table shows the details of the experiment and gives a survey of the results achieved:

Contents of the tube.	Experiment begun	Lucid after how many times 24 hrs.
Filtered Vlietwater 5 c c + Flexner	23,9, '22	After 12 × 24 hrs all still turbid, after the next sojourn of 4 × 24 hrs 28° C. all remain turbid.
" " " + Shiga Kruse	" "	
" " " + K. B. [1])	" "	
" " " + Flexner + Bacteriophage 0,1	" "	
" " " + Shiga + Bact. 0,1	" "	
" " " + K.B. + Bact. 0,1	" "	
Unfiltered Vlietwater 5 cc + Flexner	" "	4 × 24 hrs lucid.
" " " + Shiga Kruse	" "	10 × 24 " "
" " " + K. B.	" "	6 × 24 " "
" " " + Flexner + 0,1 Bact.	" "	6 × 24 " "
" " " + Shiga + 0,1 Bact.	" "	9 × 24 " "
" " " + K. B. + 0,1 Bact.	" "	6 × 24 " "

The tests of the 2nd series were conducted as follows:

The fluid of two flasks, each holding 0,5 L. of bacteriophage-containing Vlietwater, was infected with such an amount of Flexner-culture as to render it quite turbid.

To the fluid of one of the flasks 20 mgms of KCN was added, after which the flask was well fitted with a rubber stopper. Both flasks were placed at room-temperature in diffuse daylight.

After a week the fluid of the flask without KCN had become quite clear, whereas the KCN-flask still contained a turbid fluid. In the former a large number of protozoa were found, which were lacking in the latter.

On the eleventh day of the experiment the KCN flask was also getting more lucid and protozoa were noticeable in it. After a fortnight the fluid in either flask was clear.

[1]) K. B. is a Flexnerstock resistant to any bacteriophage action.

The phenomenon exhibited in the KCN flask is to be interpreted by the fact that at the beginning of the experiment the KCN destroys the vegetative forms of the protozoa and consequently they are prevented from clearing away the germs present in the water. The cysts of the protozoa are not killed by KCN. After a week so much of the KCN has been decomposed through contingent chemical processes, that the cysts again grow into vegetative protozoa, which devour the Flexner bacilli, present in the water.

CONCLUSIONS.

When summarizing our results it must be concluded that the significance of the bacteriophage for the selfpurification of water is no doubt only small. I for one did not succeed in establishing the slightest influence.

The purification is effected in the absence of the bacteriophage, whereas its presence does not accelerate the process, nor render it more complete.

The experiments again yield conclusive evidence for the prominent rôle played by protozoa in the self-purification of water.

When, under such circumstances as the laboratory enables us to establish, we eliminate the protozoa, the self-purification of water is entirely arrested even though bacteriophage be added to the water.

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