

Chapter 5

BACTERIOPHAGE INVESTIGATIONS

Early Observations

In marked contrast to the copiousness of later records, during the period of about a decade following the discovery of bacteriophage by Twort (1915) and by d'Hérelle (1917) publications on the role of bacteriophages in cholera were rather scanty.

D'Hérelle himself (1922 ; see also d'Hérelle, Malone & Lahiri, 1930), referring to his early observations in this respect, stated that :

“ Amongst about one hundred cases of cholera studied in Indochina it was possible to observe but one following recovery. In this last, in spite of daily examinations of stools, in but a single specimen taken at the beginning of convalescence has a bacteriophage active for the vibrio been found. This gave about 50 plaques when planted on agar. In spite of many attempts it has been impossible to cultivate it by serial transfers. None of the fatal cases yielded a bacteriophage.”

Continuing to work with cholera stools after the above-mentioned observations had been made in 1920, d'Hérelle (1923) was able regularly to demonstrate the presence of a lytic principle in the stools of cholera patients by

(a) incubating broth dilutions or suspensions of the faeces for about 12 hours at 37°C; (b) filtering the fluids first through kieselguhr and then through candles, and (c) after admixing agar-grown cholera vibrios to obtain a concentration of 100 million organisms per ml, incubating the mixtures for 12-18 hours at 37°C, at the end of which period the vibrios were found to have been dissolved.

The lytic principle began to become manifest in the cholera faeces 3-4 days after onset of the disease and continued to be present until the death of the patients, and in the case of the single convalescent examined, up to the end of an observation period of about two weeks. Similarly, in the case of one cholera-vaccinated individual the lytic principle became manifest on the fourth day after the first inoculation and disappeared two weeks after a second dose of vaccine had been given 8 days after the first.

D'Hérelle felt convinced that the above-described phenomena were not due to bacteriophage action, because : (1) in contrast to what was observed

in the latter case, there was a strictly quantitative relation between the concentration of the filtrates and the amount of cholera vibrios per ml they were capable of dissolving, filtrates diluted to 50% lysing only 20-25 million organisms per ml, and those diluted to 10% exerting no action even upon small inocula; (2) serial transmission proved impossible; and (3) the characteristic plaque formation remained absent when suspensions of cholera vibrios in active stool filtrates were spread on agar plates. D'Hérelle was inclined, therefore, to ascribe the observed vibriolysis to the action of a bacteriolytic ("diastatic") ferment.

It is interesting that afterwards a similar claim was made by Bernard & Guillemin (1933a), who stated that they had obtained by a method suitable for the extraction of diastases from bacteriophage-free broth cultures of *V. cholerae* a substance endowed with transmissible lytic power. As these two workers formulated it in a second note (1933b), this substance had "the characters of an activator of the diastase which in normal cultures produces an autolysis of vibrios". However, even if acceptable at face value, these observations cannot invalidate the now generally accepted, because fully confirmed, belief in the virus nature of the bacteriophages.

D'Hérelle's initial observations, which testified to the occurrence of true bacteriophagy in the case of cholera, found early confirmation through Jötten (1922), who in one instance demonstrated specific phage activity of a filtrate derived from a cholera stock strain. Flu (1923), on the contrary, obtained entirely negative results with the filtrates from 13 cholera strains which had been subcultivated monthly after they had been isolated seven years previously in Java.

Similarly Ciuca (1923) found the filtrates obtained from the faeces of five cholera patients (four of whom recovered) active only against dysentery bacilli and *E. coli*, but not against the 17 *V. cholerae* strains tested. However, as stated by Petrovanu (1924a), Ciuca and he had afterwards been able to demonstrate a weak lytic action of cholera stool filtrates on heterologous strains of *V. cholerae* not directly isolated from patients.

Meissner (1924), making "incomplete" Pfeiffer tests by intraperitoneally injecting guinea-pigs with lethal doses of *V. cholerae* and small amounts of cholera-immune serum, found that filtrates of the peritoneal exudate of such animals displayed a typical bacteriophage activity which was serially transmissible. Working obviously with phage-contaminated materials, she obtained identical results with the peritoneal exudates of guinea-pigs intraperitoneally injected only with nutrient broth.

In contrast to the findings of Meissner, Petrovanu (1924a) was unable to demonstrate a lytic action of the peritoneal exudate of rabbits injected intraperitoneally with lethal or non-lethal doses of *V. cholerae*. However, Petrovanu (1924b) found that filtrates from the washings of ground-up small intestines of cholera-immunized rabbits exerted a typical bacteriophage action on the organisms freshly isolated from the heart-blood of cholera-

infected rabbits, though not on one of the stock strains used for the immunization of his animals. Noting these as well as further discrepant results obtained with other filtrates and/or cholera vibrios of different origin, Petrovanu maintained that these organisms were evidently apt to undergo variations, sometimes even rapid changes, in their behaviour to bacteriophages.

A valuable study of the susceptibility and the resistance of cholera vibrios to bacteriophage action was made by Flu (1924, 1925). As excellently summarized in the *Tropical Diseases Bulletin* (1926), the thesis of this worker was

“ that strains resistant to bacteriophagy are, or may be, not only resistant, but also may by their growth, especially in bouillon, bring about a steady diminution of potency of any bacteriophage which may be present : there would naturally be failure to carry on bacteriophage in subpassage, if the resistant strains got the upper hand and brought about the disappearance or inactivity of the bacteriophage.

“ This idea of the author may further be set out as a number of propositions :

“ (1) A killed lysogenic strain in association with a young growing lyso-susceptible strain in bouillon culture often furnishes a potent bacteriophage.

“ (2) Lysogenic strains cultured in bouillon often develop resistant strains with lengthening of the incubation time.

“ (3) Strains which are resistant to bacteriophage bring about with their growth, disappearance of any bacteriophage present.”

Flu pointed out that these considerations explained on the one hand why d'Hérelle, using too long an incubation period for his test specimens, had usually failed to demonstrate the presence of cholera bacteriophage, and made it clear on the other hand why Meissner, who evidently worked with a specially lysogenic strain, obtained positive results even in tests with filtrates from the peritoneal exudate of normal guinea-pigs.

Finding it legitimate for the reasons stated above to utilize again ten of the cholera strains which had given negative results in his 1923 bacteriophage tests, Flu adopted the following technique :

“ Of each of these strains the well-grown layers on four agar slants were suspended in 3 ml broth or normal saline and the suspension was mixed with sufficient anhydrous Na_2SO_4 for a solid mass to be formed. This was finely triturated in a mortar, the powder was suspended in 100 ml broth, which was then heated for one hour at 58°C . The cooled broth was afterwards distributed into ten flasks, each of which contained 100 ml of broth. Each of these flasks was then seeded with one of the test cholera strains. One obtained thus ten test series, each consisting of ten flasks.

“ After an incubation for two weeks a few ml of the contents of each flask were heated for 1 hour at 58°C and 0.1-ml quantities of these fluids were used for bacteriophage tests with each of the cholera strains under examination.” [Trans.]

Tests carried out with the aid of the above-described technique showed on the one hand that only one of the ten strains examined was markedly lysogenic, i.e., capable of exerting a lytic action on phage-susceptible

strains, and on the other hand that not all of the remaining nine strains were susceptible to lysis. In fact, as established by Flu,

"only three [of these] strains were found to give lysis under all circumstances and to lead to plaque formation on the plates and slants, and only these out of 20 strains of my stock could be used for subcultivation of the bacteriophage." [Trans.]

The strongly lysogenic strain was not only lysoresistant against the bacteriophage obtained from it, but also capable of inhibiting bacteriophage action in fluid media and to lead after repeated subcultivation in such media to a complete disappearance of the bacteriophage.

Nobechi (1926a), while unable to demonstrate the presence of cholera bacteriophages with the aid of the techniques of Jötten, Meissner, or Petrovanu, found the method recommended by Flu effective. However, Nobechi stated that he had obtained at least equally good results by simply filtering the bacteriophage-containing broth cultures.

Making cross bacteriophage tests with the 18 *V. cholerae* strains at his disposal and their broth culture filtrates, Nobechi found 3 of these strains to be bacteriophage-resistant and lysogenic, while a second group of 6 strains proved to be resistant but non-lysogenic, and a third lysosensitive and non-lysogenic. It was noted that the clarification of broth cultures of lysosensitive strains under bacteriophage influence began to become manifest after 3 hours and became maximal after 4-5 hours. It was essential, therefore, to observe the tubes continuously from the beginning of the tests, because in the case of delayed examination rapidly occurring secondary growth could mask the presence of bacteriophage. Nobechi also advised making subcultivations during the phase of maximal lysis.

Studying the mutations of *V. cholerae* under the influence of bacteriophage, Nobechi (1926b) found that these were not of a permanent character: reversion to type took place after some passages in bacteriophage-containing broth media, and the strains in question became at the same time resistant to bacteriophage action, non-lysogenic, and also spontaneously agglutinable. However, spontaneous agglutination was no longer observable when such strains were transplanted on agar.

Nobechi further found that intraperitoneal injection of guinea-pigs with 0.3-0.5 ml of bacteriophage cultures protected the animals against intraperitoneal challenge with 3-10 MLD of *V. cholerae*, the phenomena of Pfeiffer's reaction becoming manifest in such animals. Bacteriophage administration by the oral, subcutaneous or intravenous route as a rule did not protect the animals against intraperitoneal challenge. The filtrates of either lysogenic or non-lysogenic cultures also showed only slight protective power. Intravenous injections of bacteriophage did not effect a disappearance of the organisms from the gall-bladder of experimental animals which had become carriers of *V. cholerae*.

In his work *The bacteriophage and its behaviour*, d'Hérelle (1926), besides referring to his own previous experiences and to those of some of

the other workers mentioned above, recorded only one observation he had made more recently :

“ In 1924, during the return of the pilgrims from Mecca, while they were quarantined at the lazaretto of Tor, I isolated from the stools of one of these pilgrims (although he showed none of the symptoms of cholera) a vibrio which agglutinated with an anti-cholera serum to its titre . . . While held in the lazaretto the stools of this pilgrim were examined every 48 hours. After a time the stool revealed a non-agglutinating vibrio, and after a further 48 hours all the vibrios had disappeared.

“ The vibrio susceptible to agglutination was bacteriophaged perfectly by the bacteriophage isolated by Flu; while the vibrio which was inagglutinable, isolated 48 hours later, was refractory. At this time the intestinal contents contained a bacteriophage having a virulence for the agglutinable vibrio.”

Interesting as this observation is, the information supplied by d'Hérelle is too scanty to show whether the “ inagglutinable ” vibrio found after the disappearance of the specifically agglutinable organisms was a rough cholera vibrio. It is quite possible, therefore, that the appearance of not specifically agglutinating vibrios in the stools of the pilgrim was of an accidental nature.

To complete the present record, reference has to be made again to the observation of van Loghem (1926), already quoted in the preceding chapter, that bacteriophage action hastened the liberation of the endo-haemolysin of *V. cholerae*.

Later Investigations

A new epoch in the history of the subject presently under review began in the year 1927 when large-scale investigations on cholera bacteriophagy were started in India under the auspices of the Indian Research Fund Association by d'Hérelle and co-workers (see d'Hérelle & Malone, 1927) and also by Morison (see Morison & Vardon, 1929). While, as will be described below, Morison and his colleagues continued to make important contributions to the knowledge on cholera bacteriophages and their role in the prevention and cure of the disease, d'Hérelle's work was taken over in 1928 by Asheshov (see Asheshov et al., 1930) who published final reports on the experiences made by him and his staff in 1933 (see Asheshov et al. 1933).

The main results obtained by these and some other investigators working in India or elsewhere, as far as they fall under the scope of the present disquisition, may be discussed under the following headings.

Types of cholera bacteriophages

In their final report (Part II), Asheshov et al. (1933b) thus described the properties of the three cholera bacteriophages they had detected early

in their work (Asheshov et al., 1930) and of two additional phages found by Pasricha, De Monte & Gupta (1932a):

“Ch ϕ A (i.e. cholera phage A) is a quick acting bacteriophage: the best race of this can produce complete lysis of vibrios in less than two hours. Its generation period is approximately between 45 minutes and 1 hour 15 minutes.

“The lysis is never permanent and is quickly followed by abundant secondary growth resistant to all bacteriophages of the type A. It attacks only the smooth elements of the culture, not touching the rough. The members of this group vary widely in activity, range, virulence and stability. The virulence can be exalted, particularly if bacteriophage is freshly isolated. But the great majority of the freshly isolated Ch ϕ A are very unstable. They die out within a very short period—sometimes within a few days—unless they are adapted to laboratory conditions by frequent transfers. The range of virulence of some of the races of this type is often restricted to a small number of strains of vibrios, but they are comparatively easily adapted to act on the other strains of smooth cholera vibrios. They do not attack the non-agglutinable vibrios even if the latter are smooth.

“Ch ϕ B. The generation period of this bacteriophage on a smooth-rough culture is usually between 1 hour 15 minutes and 1 hour 45 minutes, and the lytic action of even the most active of this type is considerably slower than that of Ch ϕ A. The lysis is seldom produced in less than three hours. The lysis is also not permanent and is followed by the secondary growth which appears later than with the type A. On the other hand, Ch ϕ B is considerably more stable than Ch ϕ A. The range of virulence is very wide . . . Ch ϕ B acts on both smooth and rough elements attacking also some of the non-agglutinable vibrios.

“Ch ϕ C is a slowly growing bacteriophage, with generation period of 2 hours to 2 hours 30 minutes. It produces appreciable lysis only with rough cultures and even then seldom complete and followed by the usual secondary growth . . . Ch ϕ C acts on the culture better on the agar surface than in the broth. The range of activity of this type is very wide: we have not yet met a strain of cholera vibrios which is not acted upon by our Ch ϕ C. It attacks also many non-agglutinable vibrios.

“Ch ϕ D (Pasricha). The generation period of this bacteriophage . . . is 1 hour 20 minutes to 1 hour 30 minutes on a smooth and 1 hour 30 minutes on a rough strain. The lytic action is slower than that of Ch ϕ B but quicker than that of Ch ϕ C; it gives an incomplete lysis in about five hours . . . The range of activity and stability of this bacteriophage have not yet been sufficiently studied.^[1]

“Ch ϕ E (Pasricha). A very slow acting bacteriophage with generation period on rough culture of 1 hour 40 minutes to 1 hour 50 minutes seems to act only on rough elements. The lytic action is very slow, but is more pronounced than with Ch ϕ C: the lysis of rough culture is more complete than with Ch ϕ C and the secondary growth appears with difficulty.”

Important additional information on these cholera phages, particularly the first three types, was supplied by Morison (1932), Rao (1932), and White (1936b, 1937).

Morison (1932) stated that cholera vibrios, if made resistant to type A cholera phages, became as a rule, though not invariably, rough in character, and also drew attention to the fact that, though the strains isolated from patients were usually smooth in character, batches found at the end of one Calcutta outbreak had been mostly rough and at the same time

¹ According to Pasricha, De Monte & Gupta (1932a), the cholera D phage had a greater range of activity than any other cholera phage.

resistant to cholera A phages. However, while admitting that these findings were in accord with those made by d'Hérelle (1926), Morison stated that he was "not yet in a position to comment on the claim that resistant strains are avirulent".

In Morison's experience cholera strains, which had become rough and resistant to type A cholera phages, became apparently changed into smooth strains if they were made resistant also to type B or type C phages. Thus, as this worker put it, the type A cholera phage seemed "to be a factor in producing rough and B and C factors in producing smooth strains".

Rao (1932), studying the relationship between the action of cholera phages and the reaction of the media used for such work, reached the conclusion that:

"Within the optimum limits lysis of the type A cholera bacteriophage is enhanced by increasing acidity and that by type B by increasing alkalinity."

Rao maintained, therefore, with much reason that differences in the reaction of the media used might exert an influence upon varying results obtained in cholera bacteriophage work.

White (1936b) stressed that the cholera phage A exerted a lytic action solely upon classical cholera and El Tor strains. Positive reactions obtained with such strains were, therefore, of diagnostic importance, but it deserved attention that some of the type A phages failed to attack Inaba-type strains of *V. cholerae*. Another important fact was that alone among the cholera phages known to White the A-type phages lysed only the S form of the vibrios they were capable of attacking, but not the R races isolated from the smooth strains without the intervention of bacteriophage. In analogy with this observation the secondary growths resulting from the action of cholera A phages were "essentially if not always entirely rough".

Further studying two cholera A phages he had obtained from Morison and from Asheshov respectively, White (1937) found that both these phages attacked all Ogawa-type cholera strains regardless of their geographical origin. Indian Inaba strains were attacked by Asheshov's A phage but not by that of Morison. Neither of these phages could be cultivated either in peptone water or on agar on the Chinese or Japanese cholera strains at White's disposal, unless the activity of the phages was enhanced by the addition of egg-white ("lysozyme") to the cultures in question.

In order to turn attention to the discovery of cholera phages additional to those enumerated above, it has first to be stated that Pasricha, De Monte & Gupta (1932b), finding a bacteriophage, which had been grown originally on a cholera-like vibrio, capable of attacking the secondary growths produced through the action of the hitherto known cholera phages, classed this new type as cholera phage "F". Further, as summarized by Pasricha and colleagues (1936): (1) Morison (1933; see also a preliminary remark made by

this worker in 1932) reported on the isolation of three new cholera phage types "G", "H" and "J"; (2) Pasricha (1933) found a "K" type, active for rough vibrios only (White, 1937); (3) an "L" type was reported in 1935 by Anderson; and (4) an "M" phage had been recently isolated from a vibrio strain not agglutinable with cholera-immune serum which had been cultivated from the faeces of a cholera patient. As noted by Pasricha and colleagues (1936), this new phage, while slow in action, had a wide range of activity, attacking some cholera-like vibrios as well as most cholera strains.

Commenting upon these observations, the same authors (1936) emphasized that among the cholera phages known thus far the A phage alone was restricted in action to vibrios agglutinable with cholera-immune serum and acted, moreover, solely on organisms possessing a considerable degree of smoothness. The cholera phage A was also immunologically in a class of its own, because sera raised with it inhibited only the action of their homologous phage, whereas serological relationships existed in the case of the other cholera phages.

As stated by White (1937), he proposed to continue work with the L cholera phage. However, he lost the strain which had been isolated originally by Anderson (1935) from Calcutta sewage, and a second strain labelled phage L received afterwards was found incapable of lysing the cholera strain attacked by the original L phage. White considered it advisable, therefore, to call the second strain at his disposal the "LL" phage, thus indicating that it was probably distinct from the original type. Summarizing his experiences with this second strain, White stated that the LL phage

"is possibly the most frequently occurring of Indian cholera phages. The majority at least of cultures of *V. cholerae* from Indian sources are LL-lysogenic. On the other hand those Chinese and Japanese strains of *V. cholerae* which have been so far examined have been found LL-free and LL-sensitive. While all *V. cholerae* examined have been found either infected with or sensitive to LL, neither of these conditions has yet been detected in El Tor and other vibrios."

While, as will be seen below, the LL phage does not quite show the limitations in geographical distribution postulated by White, the observations of this worker on the restriction of the distribution of this phage to the classical *V. cholerae* are of great interest.

Another interesting fact established by White was that the usually feeble lytic action of the LL phage could be enhanced by the addition of "lysozyme" (i.e., egg-white in a 1:25 concentration) to the culture media. As already alluded to, White also found that two A-type cholera phages, which failed to multiply on Far Eastern Inaba strains in spite of their specific affinity for the polysaccharides of these strains, attacked the organisms in question vigorously in the presence of the egg-white lysozyme. Where no such affinity existed, the lysozyme failed to promote

bacteriophagy. In White's opinion this observation supported the view "that the combining property of bacteriophage is to be clearly distinguished from its lytic activities".

In apparent agreement with this view White found that, though no signs of bacteriophage action became manifest when *rough* LL-free races of *V. cholerae* were exposed on agar plates to the action of this phage, it could multiply indefinitely upon the R cultures which, like the smooth growths, became lysogenic. He added that:

"Cholera phage LL is fixed and inactivated by the polysaccharides of S,R and ρ *V. cholerae* and probably has a specific affinity for the substance which I have termed C γ (White, 1936[a])."

Continuing work with the LL phage, Pasricha, Lahiri & De Monte (1941) established that (a) the secondary cultures obtained after the action of this phage were lysable by the 12 other types of cholera-phages (A \rightarrow M), while (b) the LL phage acted reciprocally on the secondary cultures resulting from the action of each of the other types of cholera-phages. White's assumption that the LL phage was a new type being thus confirmed, Pasricha and co-authors proposed for it the name of cholera-phage "N".

Like cholera-phage A, the N type exerted no action on cholera-like vibrios. Evidence of its presence could be found in only 3 out of 115 recently isolated cholera strains but it is noteworthy that these had all been obtained within a short time from one and the same locality. Positive results were obtained with all Indian stock cholera cultures and, in contrast to White's experiences, also with some strains from Hong Kong, three of which yielded N phage, while a fourth proved resistant and a fifth sensitive to the action of this phage. In confirmation of White's observations, Pasricha and colleagues found egg-white lysozyme an excellent means of propagating the N phage but they obtained satisfactory results also with solid or semi-solid agar.

In their article describing the D and E types of cholera-phages, Pasricha, De Monte & Gupta (1932a) also stated that they had isolated from the water of the Hooghly river a "W" phage, found to be capable of lysing a number of cholera-like strains. Though the latter had proved to be resistant to the action of cholera-phages A \rightarrow C, the secondary growths appearing after the action of W phages were found to be lysable by cholera-phages.

Subsequently Pasricha and colleagues (1932b) referred to the isolation of 8 (or, to judge from their protocols, rather 9) strains of *vibriophages*, found to be active against cholera-like strains which were not lysed by cholera-phages. After repeated passages on their respective vibrios, 5 of these phages acquired the property of lysing cholera vibrios. However, as stated by the same authors in 1936, such *vibriophages*, "if active on agglutinable (i.e. cholera) vibrios do not give the reciprocal cross test".

To judge from a short remark made in 1934 by Russell (1935), up to then over 30 different races of vibriophages had been isolated in India.

Pasricha, De Monte & Gupta (1931a), studying the seasonal incidence of cholera phages in Calcutta, found that:

“(a) Cholera phages in Nature vary with the incidence of the disease. It is rare to isolate cholera phages from water during the non-cholera season.

“(b) The mortality rate which is high at the beginning of the cholera season, falls rapidly when cholera phages have become widely distributed in Nature. The spread of bacteriophages thus apparently plays a very important role in the lowered mortality, and in bringing an epidemic to a close.

“(c) Cholera phages in Nature are of the quick-acting type A, and evidence is presented suggesting that the types B and C die out in Nature.”

These conclusions are in accord with the postulations of d'Hérelle and his co-workers, to which reference will be made later. They stand, however, in a curious contrast to the contention of Morison (1935) that, unlike other phages types, cholera A phages, though frequently met with in the patients, “may have little effect on the infectivity or virulence” of *V. cholerae*.

Fixation and inhibition of cholera phages

White (1936b) pointed out that observations by several workers, made with bacterial species other than the vibrios, had demonstrated the power of specific extracts separated from the organisms in question to fix and inactivate particular bacteriophages. He made, therefore, corresponding tests with substances extracted from vibrios and cholera phage types A→J. White was thus able to show that

(1) the A-type phage was selectively inactivated by the smooth specific polysaccharide of cholera and El Tor vibrios ;

(2) the polysaccharides derived from cholera-like vibrios as well as those from R and ρ races of *V. cholerae* exerted no inhibiting action on the A-type cholera phage;

(3) “lipoid” constituents of the cholera vibrios, obtained by alcohol extraction, while in properly conducted tests not interfering with the action of cholera phage A (and also of the type D), specifically inhibited the phage types C, E, G and H, while the B-type phage was not invariably inhibited by these substances.

White concluded from these observations that, regardless of whether the polysaccharides or the lipoids specifically bound the phages, “in both cases the resistance of the secondary culture is probably due to its loss of susceptible substance rather than to any positive modification or ‘immunity’”.

Pandit, Maitra & Datta Roy (1936), who also made an early study of the problem presently under review, used the following technique to prepare extracts of cholera and other vibrios:

“The organisms were grown in Roux's flasks for 48 hours. The growth from each flask was washed and emulsified in 10 c.c. distilled water. The emulsions

were placed in a water-bath at 55°C for 72 hours. They were then diluted by adding twice the amount of distilled water, and filtered through a Seitz filter."

Applying this technique, Pandit and co-workers tested the inhibitions produced by the extracts of 17 strains of cholera, El Tor, and cholera-like vibrios on cholera-phages A→K. It was found that the phage types C and G were not inhibited by any of the extracts tested and that, on the other hand, the extracts of two cholera-like vibrio strains as well as that of the single available El Tor strain did not inhibit the action of any of the above-mentioned bacteriophage types.

No definite correlation appeared to exist between phage-type inhibition and resistance of the strains to the phages in question, but multiple-type resistance was found to be associated with a diminution in the number of types inhibited. A parallelism was found to exist between the types of phage inhibition produced by the extracts of the strains and the polysaccharide content of the organisms concerned according to the classification of Linton and his co-workers (see Chapter 3).

Continuing the above-described work, Maitra (1939) combined phage-inhibition tests (types A→L) with precipitin tests, made with sera raised against strains falling into different groups according to the type of their phage inhibition and polysaccharide content. It was found that according to the results obtained by such combined tests the vibrios could be divided into two groups, namely, one consisting of typical smooth cholera vibrios and El Tor vibrios in the strict sense, and a heterogeneous group comprising atypical cholera vibrios, particularly rough strains, and cholera-like vibrios. Maitra concluded in contrast to the statements of Pandit and colleagues that:

"Both the inhibition and precipitin reaction appear to depend on a common factor which is related to the complex polysaccharide receptor of the cholera vibrio but bears no direct relation to the type or quality of polysaccharide by chemical analysis as found by Linton."

As stated in a preliminary note by Doorenbos & Cossery (1950), a serum raised in rabbits against cholera B phage, besides possessing weak agglutinating properties (titre 1 : 300), inhibited in dilutions of 1/1000 after an incubation for 4 hours the activity of this phage.

Action of cholera-phages on El Tor vibrios

Though repeated references are made elsewhere in the present chapter to the behaviour of El Tor vibrios under the action of cholera-phages, it is necessary to deal separately with the observations made in this respect by Jadin (1936).

Making comparative tests with a cholera bacteriophage, Jadin found it far more active for classical cholera than for El Tor vibrios: the former organisms were completely lysed when the phage was used at a dilution

of 10^{-8} , and after joint subcultivation for several months even at a dilution of 10^{-10} . The El Tor vibrios, though slightly lysed by 10^{-5} bacteriophage dilutions, were completely lysed only by 10^{-3} dilutions. Subcultivation of the phage on cholera vibrios did not increase its lytic power for *V. El Tor*; subcultivation of the phage on the latter organisms did not abate its potency for *V. cholerae*.

Heating of the bacteriophage dilutions for half an hour at 64°C did not deteriorate their lytic action for cholera vibrios, but such heated dilutions produced no lysis of El Tor vibrios. However, it was possible to restore the activity of such phage dilutions for the *V. El Tor* by repeatedly exposing them to a temperature of 75°C .

A serum raised with a bacteriophage active for cholera vibrios but inactive for *V. El Tor* was found to inhibit the action of unheated cholera phages on the latter organisms, but only to diminish the phage activity against *V. cholerae*. The serum completely inhibited the action of cholera phages heated to 65°C .

It was further established that repeated subcultivation of an El Tor strain contaminated with a 10^{-8} dilution of bacteriophage rendered the latter capable of lysing El Tor vibrios, apparently because in the course of subcultivation a multiplication of the phages had taken place.

Jadin concluded from these observations that the different behaviour of the cholera phage towards cholera and El Tor vibrios respectively was the result of differences in the phage sensitivity of the two organisms and not of the separate presence of an anti-cholera and an anti-El Tor factor in the phage.

Bacteriophage-produced vibrio variations

Reporting on their studies in India, d'Hérelle, Malone & Lahiri (1930; see also d'Hérelle, 1930) stated that under the influence of bacteriophages the vibrios underwent profound changes: the motility of the organisms was apt to become eventually lost; their morphology became changed, bacillary and ultimately coccoid forms making their appearance; the reactions produced by the vibrios in carbohydrate-containing media became modified, and the property of reducing nitrates became lost. More important still, bacteriophage action led to a loss of the specific agglutinability with cholera-immune sera and also to a loss of virulence.

Commenting upon their findings in this respect with the faeces of cholera patients, d'Hérelle, Malone & Lahiri stated it to be "beyond dispute" that these changes in the properties of the organisms, which had been observed as well under the action of bacteriophages *in vitro*, "constitute real mutations of the typical cholera vibrio". They maintained in this connexion that:

"When the loss or the modification of a character is the result of a mutation, this character apparently cannot be recovered. The mutation appears to be final and the return to the original form impossible, in contra-distinction to what is produced when the loss of a character is simply the result of 'disuse'.

“ In a word, if a powerful bacteriophage is brought into contact with cholera vibrios, the latter are parasitized and then rapidly and finally destroyed. If the bacteriophage which attacks them is less potent a certain number of vibrios resist but *contract a chronic disease*, transmissible to their descendants, which has the effect of modifying, more or less profoundly, their characters.”

While the farguing conclusions which d'Hérelle and his colleagues drew from the above and allied observations will receive attention later, the following records of other investigators deserve consideration at the present juncture:

Finkelstein (1931) drew attention to unpublished observations according to which the phage-resistant growths appearing after the action of a cholera phage fell into two types in regard to their agglutinability, one showing a granular and the other a large-flake agglutination. This dissociation, which appeared to be stable in character, was accompanied by a loss of the motility of the organisms.

Chen (1932), testing two cholera strains with the aid of a cholera-dysentery phage, noted the successive appearance of three types of variant colonies, two of which showed signs suggestive of roughness, producing a granular growth in broth subcultures and showing spontaneous agglutination in saline suspensions. Motility was found to have disappeared in the case of one of these variants and also in that of the third, apparently smooth, variant. All three variants reverted to type after more or less prolonged subcultivation, more rapidly after animal passage. While the variants showed no marked changes in agglutinability, they seemed to be less virulent for hamsters than the parent strain.

These observations were supplemented by valuable findings made by Chen (1933) with 195 stool specimens of 21 cholera patients, 18 of whom could be studied throughout their convalescence. Chen found that the cholera colonies thus isolated

“ were divisible into 3 distinct types according to their ‘ smooth ’ and ‘ rough ’ reaction, susceptibility to cholera-phage and agglutinability with high titered serum. Smooth type, lysible colonies by cholera-phage with full titer agglutination was always isolated in abundance during early acute stage of the disease when cholera-phage had not yet appeared . . .

“ As soon as cholera-phage appeared in the stool and the patient was convalescent, smooth type colonies became scanty or entirely absent, and intermediate and rough resistant variants of comparatively low titer agglutination (1:160-1:640) took their place . . . Motility of the rough variants was also sometimes impaired and non-motile colonies of rough cholera vibrio were isolated in 4 cases. The motility, however, could be restored on subsequent transfers in laboratory media.”

In the case of five of the above-mentioned 18 patients, absolutely “ non-agglutinating ” cholera-like vibrios appeared, which proved to be rough in tests with Millon's reagent and cholera phage-resistant. However, cross-agglutination and absorption tests with sera raised against (a) smooth cholera vibrios, (b) slightly agglutinable rough variants, and (c) four of

the cholera-like vibrios respectively showed a full antigenic independency of the latter organisms, not only as far as the smooth cholera vibrios, but also as far as the rough variants of the latter were concerned. It was in accord with these observations that daily transfers in alkaline peptone water, repeated for three months, as well as animal passages, while producing a tendency to better agglutinability with cholera-immune sera in the case of the rough variants, yielded no such results in the case of the cholera-like strains.

An interesting study of the antigenic characters of three types of secondary cultures obtained with A, B, and C cholera phages respectively, was made by Pandit & Rao (1932) who thus summarized their results:

“The main change was in respect of presence of somatic antigen. This antigen was considerably diminished in the case of secondary A but it persisted in varying amounts with secondaries B and C. The above gradation was noted in the case of flagellar antigen also. The power to provoke normal agglutinins was found to be proportional to the amount of somatic antigen present.”

It is important to note that the rough variants of *V. cholerae* produced independently of any phage action “showed more or less the same antigenic structure as was noted with A phage type secondary cultures”. The variants appearing after action of cholera phages B or C could not be considered as truly rough in character because, as noted above, they continued to contain some of the smooth somatic antigen.

As already referred to in Chapter 4, Morison (1932) stated that he had been unable to make cholera vibrios specifically inagglutinable by growing them in the presence of bacteriophages; he added that, when working under conditions that precluded contamination, he and his colleagues had also been unable to transform true cholera into cholera-like vibrios by other means.

These observations were confirmed by Vardon (1940), who stated that the secondary growths, which appeared after the individual action of the 11 types of cholera phages known in 1935 on a typical *V. cholerae* strain, were agglutinated to over 50% of the titre by an H + O serum raised against this parent strain. The sugar reactions of the secondary growths were also identical with those shown by the original culture.

However, Morison reported in 1935 that, using combinations of the cholera phages instead of single types, he and his co-workers had been able to effect

“changes in the morphology, the colonies on agar, the growth in broth, the salt stability, the agglutinability and the ability to ferment sugars which varied with the combinations of types of bacteriophage and the period of action. These changes ranged from slight to so profound that the bacteria resulting therefrom are quite unrecognizable as vibrios.”

Some details on these and on analogous observations made during the following years with the aid of O-agglutinating sera raised against cholera and phage-produced variant strains were furnished by Vardon (1940).

Though he found *in vitro* some changes in the serological behaviour of such variants, it is noteworthy that according to a statement made by Anderson (1937), the presence or absence of phages infecting the vibrios in nature did not appear to influence their agglutinability.

Careful studies on the changes 16 typical, non-haemolytic and more or less phage-sensitive Indochinese cholera strains were apt to undergo under the influence of cholera bacteriophage were made by Bernard & Liang (1933) and by Bernard, Raynal & Liang (1933). As stated by Bernard & Liang, the secondary cultures obtained from these 16 strains after the action of a cholera phage from Assam showed the following properties:

(a) while no change in agglutinability was observable in four instances, six of the variant strains showed an impairment and six a total loss of their agglutinability with the three cholera-immune sera used;

(b) the proteolytic properties of the variant strains were not altered in six instances, whereas the others liquefied gelatin and coagulated serum with some delay and to a lesser degree, or were even altogether unable to liquefy such media.

As shown in the case of one of the strains, daily subcultivation of the phage-produced variants on gelatin-agar led to a gradual restoration of the properties of the parent strain, which became complete after the seventh passage. If such a "purified" strain was again contaminated with bacteriophage, the cycle of modifications was started once more. Bernard & Liang maintained in this connexion that

"the isolation from the seven first passage cultures on gelatin-agar of colonies which had preserved an absolutely normal form shows that pure and modified colonies co-exist in the same growths. In the course of successive passages, the pure colonies develop more rapidly than the contaminated colonies and restore, after a minimum of seven passages, the original culture. There can be no doubt that, depending upon the character of the original culture and the activity of the bacteriophage used, marked differences ought to exist in the number of passages necessary for the reconstitution of the growths to their original state." [Trans.]

Bernard, Raynal & Liang (1933) supplemented these observations by stating that the cholera-red reaction, positive in the case of all 16 original strains, was more marked in that of six of the secondary growths appearing after bacteriophage action, and weaker than normal four times. The findings made by these workers regarding the behaviour of their strains in blood-containing media will receive attention in a later part of this chapter.

In order to assess the alterations of the properties of cholera vibrios under the influence of bacteriophage, Damboviceanu, Combesco & Soru (1934) used six strains, two of which were of a smooth character, while two were rough and two were either in a transitory stage or consisting of a mixture of smooth and rough elements. Bacteriophage action produced no alteration of the specific agglutinability of all these strains and also did not alter the physico-chemical properties of the two rough strains. However, the variant growths developing after bacteriophage action in the case of the

smooth and transitory strains showed changes in their agglutinability with tryptaflavin and in the zones of their acid agglutination as well as in the speed of their cataphoresis, exhibiting in these respects, in contrast to the original growths, the reactions characteristic of R strains.

Damboviceanu & Soru (1934), studying the content in residual antigen of three smooth, two transitory, and one rough cholera strains before and after bacteriophage action, found that

(a) residual antigen, though abundant in the extracts of the smooth parent strains, was no more demonstrable in the growths which had recently acquired rough properties under bacteriophage action;

(b) however, strains which already exhibited rough features before bacteriophage action yielded after as well as before this action extracts as rich in residual antigen as the smooth strains or even showed a higher content in residual antigen.

A study of the rough variation of *V. cholerae* and its relation to resistance to cholera A phage by Yang & White (1934) led to the following conclusions of importance for the subject presently under review :

“(a) According to the condition of the ‘ultrapure’ culture of *V. cholerae* exposed to A type cholera phage the resistant growth is predominantly smooth, intermediate or rough in serological character;

“(b) Extreme rough variants isolated from ultrapure cultures without the help of A cholera phage are identical with those obtained by its use and seem to be invariably resistant to this agent;

“(c) Attempts to isolate from ultrapure cultures, by methods of simple selection, variants resistant to A type phage have met with a single but apparently significant success. On the whole we are inclined to believe that resistance to A phage is not a modification induced by phage action but that resistant elements are present in the ultrapure culture and survive lysis.”

As recorded by White (1937a), the action of the LL cholera phage resulted

“in a very variable and usually trivial tendency towards roughness in the surviving growth, which may lead to its behaviour as a mixed SR antigen, to some reaction with R agglutinin and even in certain cases to somewhat increased susceptibility to the precipitating action of NaCl”.

The observations made in regard to the problem presently under review by Doorenbos (1932) and Scholtens (1935) as well as part of those by Bernard, Raynal & Liang (1933) deserve separate consideration, because these workers paid particular attention to the modifications produced by the action of cholera phages in the behaviour of the vibrios in blood-containing media.

Doorenbos (1932) reported in this connexion that he had worked with 70 cholera strains which were originally lysosensitive and incapable of lysing goat erythrocytes. After having been artificially contaminated with bacteriophage, 14 of these strains became strongly haemolytic. Though finding that these haemolytic variants were not stable, their subcultivation leading to the appearance of non-haemolytic as well as of haemolytic colonies, Doorenbos felt entitled nevertheless to claim an *in vitro* transformation of cholera into El Tor vibrios.

As found by this worker, bacteriophage action led also to other modifications of the properties shown by the parent strains : while the colonies of the latter were endowed invariably with marked haemodigestive power, after bacteriophage action this property became less conspicuous or was even no more apparent. Further, phage-contaminated strains showed a less abundant, sometimes a granular growth in peptone water. The cholera-red reaction, though markedly varying, was as a rule more strongly positive in the case of the phage-contaminated strains than in that of the parent strains. The degree of gelatin-liquefaction produced by the phage-contaminated strains showed more marked variations than was the case with the parent strains; sometimes the phage-contaminated growths failed to liquefy gelatin.

Testing 35 of his strains with two different cholera-immune sera, Doorenbos found that in the case of the parent strains agglutination took place both in the form of flakes and of granules. Several of the lysogenic growths, on the contrary, showed merely agglutination in the form of granules, which sometimes were hardly visible with the naked eye. One of the two sera, which had a low titre, sometimes failed altogether to agglutinate colonies of the lysogenic strains.

The general conclusion which Doorenbos reached on account of these observations was that

“ it is possible (namely, through the intervention of d'Hérelle's bacteriophage) to transform *in vitro* the agglutinable vibrio into a non-agglutinable vibrio, a non-haemolytic vibrio into a haemolytic vibrio, a vibrio giving a feeble indol reaction into one giving a strong reaction : to isolate, from one and the same strain agglutinable and inagglutinable vibrios, haemolytic and non-haemolytic vibrios ”. [Trans.]

While most of these postulations of Doorenbos are acceptable because they are in agreement with corresponding observations made by other workers, exception must be taken to the claim that he had succeeded in transmuting cholera into El Tor vibrios. For all that he was able to achieve in this direction was to produce quite unstable haemolytic variants of *V. cholerae*, whereas actually, as has been shown in the third chapter, the reactions given by cholera and El Tor vibrios respectively in properly conducted haemolysis tests are characterized by a great stability. There can be no doubt, however, that, as had been shown previously by van Loghem (1926) and was confirmed by the observations of Doorenbos as well as by the findings of Bernard, Raynal & Liang (1933) and Scholtens (1935) recorded below, the action of cholera-phages is apt to exert an activating influence on the latent haemolytic properties of *V. cholerae*.

The experience, in this respect, of Bernard and co-authors (1933) with 16 cholera strains may thus be tabulated:

Reactions	Parent strains	Secondary growths obtained after phage action	
		for 6 hours	for 24 hours
Haemagglutination (sheep erythrocytes)	uniformly positive	identical, 10 times stronger, 5 times weaker, once	identical, 11 times stronger, 4 times weaker, once
Haemodigestion (rabbit-blood agar)	uniformly positive	identical, 9 times weaker, 7 times	identical, 7 times weaker, 9 times
Haemolysis (saline suspensions of sheep erythrocytes)	totally negative even after 48 hours	4 times positive after 24 hours, 9 times inconstant results	

Disquieted by the possibility of diagnostic errors which might be made if haemolytic variants of *V. cholerae* were met with in actual practice, Bernard and colleagues recommended resorting to serial subculture of possibly modified growths so as to obtain once more colonies of a typical character.

Scholten (1935) inoculated 15 tubes containing 0.5% suspensions of sheep erythrocytes in broth with isolated colonies of a secondary growth of *V. cholerae* obtained after phage action. Slight but definite haemolysis was noted in those tubes in which, undoubtedly as the result of roughening, a flocculent growth took place, while haemolysis was almost negligible in the tubes showing a diffuse growth, characteristic of smooth organisms. The variants definitely producing haemolysis were found to be phage-resistant but non-lysogenic.

This demonstration of a potentially existing parallelism between S-R transition and aberrant haemolytic reactions given by the vibrios in question is of considerable interest.

While several of the above-quoted observers, including Doorenbos, found that under the influence of bacteriophages the specific agglutinability of *V. cholerae* might be impaired or even lost, it should now be mentioned that a few workers claimed that strains of this organism, which were no longer amenable to the action of cholera-immune sera after they had been exposed to phages, might regain their original agglutinability and that even hitherto "inagglutinable" cholera-like vibrios might after phage action show serological reactions identical with those of the true cholera vibrios.

As already partly discussed in Chapter 4, claims to this effect have been made by Pasricha, De Monte & Gupta (1931b, 1932b, 1933).

In their 1931 paper, these workers recorded that the lysability of 355 strains of cholera-like vibrios of various origin by cholera-phages was as follows :

Source	Number of strains tested	Type A	Type B	Type C	Type B and C	Percentage lysed
River and tank water . . .	88	—	2	26	18	52.0
Stools of healthy persons .	82	—	—	—	13	16.0
Stools of cholera convalescents	126	—	8	12	4	19.0
Totals	296	—	10	38	35	23.0

Note : No instance of lysis was observed in the 59 specimens of cholera patients' stools, the vibrios isolated from which were frequently found to be contaminated with type A cholera-phages.

As noted in the preceding chapter, Pasricha and colleagues found that some of the secondary phage-resistant colonies which developed after action of cholera-phages B, C, or B and C on these cholera-like strains were agglutinable by a high-titre cholera serum, completely absorbed the agglutinins from this serum, and were capable of producing sera which agglutinated

cholera vibrios at high titre. As also noted, Pasricha and his colleagues, even though finding this acquired agglutinability difficult to maintain, ascribed great significance to their observations, feeling convinced that a large proportion of the "non-agglutinable" vibrios present in cholera-affected localities were mutant forms of *V. cholerae*, apt to play a considerable role in the causation of the disease. However, discussing the origin of the phage-resistant and specifically agglutinating vibrios obtained after bacteriophage action on cholera-like vibrios, these workers admitted the difficulty of refuting the criticism that the apparently transmuted organisms might have been pre-existent in "a very small proportion" in the original growths. There is no doubt in the mind of the present writer that this interpretation of their findings and of some analogous results afterwards recorded by Vardon (1940) is by far the most likely one.

In their 1932 paper, Pasricha, De Monte & Gupta referred to one "non-agglutinating" strain isolated from the faeces of a patient who showed clinical features of cholera, stating that (a) the vibrios in question were lysable by a bacteriophage isolated from water, (b) the secondary growths developing after the action of this "vibriophage" were lysable by cholera-phages B, C, and D, and (c) these secondary growths were agglutinable to full titre by a highly potent cholera serum. Pasricha and co-authors added, without giving details, that similar changes were brought about by vibriophages grown on three other strains of cholera-like vibrios.

As set forth already in the preceding chapter. Pasricha and colleagues (1933) claimed that out of 56 strains of "non-agglutinating" vibrios 11 became agglutinable with cholera-immune serum after action of cholera-phages, while the same result was obtained in 13 instances after the action of vibriophages. Commenting upon these findings, Pasricha and his co-workers stated that:

"... We have not made a sufficiently extensive study of vibrios and phages in non-endemic areas, nor have we made an investigation into the seasonal variations of vibriophages in relation to the epidemic of cholera, to enable us to draw definite conclusions as to the part played by vibriophages in the epidemiology of cholera, but in the laboratory under the influence of a virus disease (cholera-phage) the typical cholera vibrio becomes a harmless saprophytic organism which when once again parasitized by another virus disease (vibriophage) assumes the characteristics which render it indistinguishable from the typical virulent type... Whether the vibrios regain their virulence or not we have no means of judging. The conclusion is forced upon us that the vibriophages play an important part in the epidemiology of cholera and that they are one of the important factors in bringing about a regeneration of degenerated cholera vibrios."

As reported by Anderson (1940), workers in the King Edward VII Memorial Pasteur Institute in Shillong, bearing in mind that cholera-like vibrios found in surface-waters might be cholera germs which had lost their specific agglutinability, tried to produce a reversion of the organisms to their original state by growing water vibrios in dilutions of antiphage sera. However, these attempts gave entirely negative results.

In a preliminary note published in 1950, Doorenbos & Cossery claimed on the contrary to have transmuted with the aid of an antiphage serum, produced by the repeated intravenous administration of type B cholera-phages to rabbits, a rough variant of *V. cholerae* Korein into the smooth form.

The two workers stated that they had used for this purpose 1/100 and 1/500 dilutions of the antiphage serum in broth, in which the rough vibrios were cultivated two times in succession for 48 hours respectively. Final subcultures were then made and cultures on agar were plated out after an incubation of the broth tubes for 72 hours. After the agar plates had been left at room temperature for 5 days, the vibrios grown repeatedly in the presence of antiphage serum showed—in contrast to those grown under identical conditions in 1/100 dilutions of normal rabbit serum—evidence of a reversion to the S type. Through subcultivation on agar slants growths of a definitely smooth appearance were obtained, which were stable in normal saline and were agglutinated by specific sera at the same titre as the original smooth cultures of *V. cholerae* Korein (the causative organism of the 1947 cholera outbreak in Egypt).

While maintaining that the action of the antiphage serum was responsible for the transmutation of rough cholera vibrios into the smooth form, Doorenbos & Cossery admitted the possibility of “a spontaneous R-S transformation of the R-vibrios when grown on different media”. They proposed to confirm the validity of their preliminary findings through further investigations, but—as far as the present writer is aware—no confirmatory publication on this subject has been made. Moreover, as has been pointed out in the preceding chapter, even final proof of such transformations under highly artificial conditions would not constitute proof that analogous reversions take place under natural conditions—a postulation which, as has been explained, appears to be altogether unlikely on epidemiological grounds.

On the other hand, the evidence adduced above leaves no room for doubt that bacteriophage action is apt to effect a transmutation of typical smooth cholera vibrios into an “inagglutinable”, evidently a rough, form.

Use of cholera-phage-produced lysates for vaccination

While, in order to avoid duplications, the use which has been made of bacteriophages in cholera diagnostic work as well as in the treatment and mass control of the disease will be described in later parts of this book, attention has to be devoted at present to some attempts to utilize cholera-phage-produced lysates for vaccination.

As summarized by Maitra & Mallick (1931), it was suggested by d'Hérelle, Malone & Lahiri (1930) that a solid and lasting cholera immunity might be produced in man by the administration of single 1-ml doses of cholera-phage lysates. Commenting upon this recommendation, Maitra & Mallick stated that it was

“probably based on reported protection against ‘Barbone’ induced in buffaloes by injections of the phage lysate of *B. bovissepticus*. The type of immunity produced by

injections of cholera phage lysate should be either antitoxic or antibacterial or a combination of both. As the bulk of bacterial bodies in a lytic filtrate undergo complete dissolution, one would naturally expect to find in it more of the disintegration products of organisms (the so-called endotoxins of vibrio in this case) than intact bacteria themselves, in addition to bacteriophage protobes which grow at the expense of these organisms. Before attributing any property of inducing active antitoxic immunity to such filtrates it must be proved that they contain potent toxins as weak toxins can never act as satisfactory antigens."

In order to test the toxic properties of cholera phage lysates and to explore at the same time the immunogenic value of such preparations, Maitra & Mallick injected three rabbits with 1- to 3-ml doses of a *V. cholerae* lysate, produced with a potent cholera phage. Careful observation of the animals for two weeks showed no impairment of their health. However, when the animals were injected intravenously on the 15th day with amounts of killed cholera vibrios equivalent to 1.5 times the calculated minimal lethal dose, like the control rabbits they developed diarrhoea, collapsed, and died in 24 hours.

Considering it possible, however, that addition of cholera phage lysates to standard cholera vaccines might enhance the immunogenic value of the latter, six rabbits were given subcutaneously two doses of cholera vaccine of 0.5 ml and 1 ml respectively, to which each time 1-ml doses of a cholera phage lysate had been added. Bactericidal tests made two weeks after the second inoculation with the sera of these animals gave results identical with those obtained in the case of a group of control animals, which had been injected only with standard vaccine doses.

As far as one is entitled to judge from these limited experiences, addition of phage lysates to standard cholera vaccines does not enhance the bactericidal power of the latter. Hence, even though it was found that the agglutination titres of the sera of the animals injected with phage lysates as well as with vaccine were on the whole higher than was the case in the control group, addition of cholera phage lysates to standard cholera vaccines offers presumably no advantages.

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