STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

IV. Concerning the Oneness of the Bacteriophage.

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D'Hérelle believes that the bacteriophage is a filterable, ultramicroscopic virus, parasitizing upon and destroying actively growing, susceptible bacteria. According to his conception, "there is but a single bacteriophage, common to both man and animals, capable by adaptation of acquiring a virulence toward all bacterial species."[1]

Evidence exists against this view. Some investigators, having failed in their attempts at adaptation of the bacteriophage,[2,3] believe in the existence of a number of serologically distinct strains, even among closely related varieties of the lytic principle.[4–8] This conception finds support in the fact that the secondary overgrowth of bacteria, appearing in the culture in the presence of lytic principle, is specifically resistant to lysis by the agent conditioning its appearance, but is often susceptible to the lytic action of another closely related lytic filtrate.[2, 5, 9, 10] Further evidence in favor of a multiplicity and relative specificity of lytic principles is to be seen in the differences in the characteristic size and appearance of plaques, or sterile spots,

[1] d'Hérelle, F., The bacteriophage. Its rôle in immunity, English translation by Smith, G. H., Baltimore, 1922, 121.

[2] Matsumoto, T., *Wien. klin. Woch.*, 1923, xxxvi, 759.

[3] Otto, Munter, and Winkler, quoted from Otto, R., and Munter, H., *Ergebn. Hyg., Bakt., Immunitätsforsch. u. exp. Therap.*, 1924, vi, 41.

[4] Wagemans, J., *Arch. internat. pharmacod. et thérap.*, 1923, xxviii, 181.

[5] Gratia, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 821.

[6] Seiffert, W., *Z. Immunitätsforsch., Orig.*, 1923–24, xxxviii, 301.

[7] Bail, O., and Watanabe, T., *Wien. klin. Woch.*, 1922, xxxv, 169.

[8] Bruynoghe, R., and Appelmans, R., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 96.

[9] Bail, O., *Z. Immunitätsforsch., Orig.*, 1923–24, xxxviii, 57.

[10] Okuda, S., *Arch. Hyg.*, 1923–24, xcii, 109.

when bacteria are grown in the presence of different lytic filtrates on the surface of agar.[10, 11]

In the course of experiments preliminary to a study of the influence of hydrogen ion concentration on the adsorption of lytic principle by bacteria, we have made observations which, in our opinion, are favorable to this view of a multiplicity of the lytic principle. It was found that the four lytic filtrates which were examined showed distinct differences in the response to changes in the hydrogen ion concentration of the medium, irrespective of the species of bacteria serving as substratum for the production of the active filtrates.

The experimental procedure was as follows:

Influence of Temperature and of Duration of Exposure.

Lytic filtrates, prepared by growing susceptible bacteria in the presence of active principle upon unbuffered broth, adjusted to pH = 7.4, were diluted 1:1000 in physiological salt solution,[12] and 0.2 cc. of this dilution was added to a series of tubes, each containing 1.8 cc. of buffer solutions, varying from pH = 2 to pH = 12 (thus diluting the original filtrate 1:10,000). These mixtures were kept at a constant temperature, and after a stated period of time 0.1 cc. of the contents of each tube of the series (containing now 10^{-5} cc. of the original filtrate) was transferred to 9.9 cc. of sterile buffered broth (pH = 7.4), and titrated by the serial dilution method.[13, 14]

Electrometric measurements have shown that the addition of 0.2 cc. of diluted filtrates to 1.8 cc. of the various buffers did not change appreciably their respective hydrogen ion concentrations. Similarly it was established that the subsequent transfer of 0.1 cc. of the mixture of various buffer solutions containing the lytic principle into 9.9 cc. of buffered broth (pH = 7.4) resulted, to all intents and purposes, in the immediate neutralization of solutions added.[15]

In the study of the adsorption of lytic principle by bacteria, to which this series of experiments was a preliminary, the tests were carried out at a temperature of +7°C., in order to avoid the rapid multiplication of bacteria and a corresponding increase in the concentration of the lytic principle during incubation. All the experiments reported in the present paper were performed at this tem-

[11] Watanabe, T., *Z. Immunitätsforsch., Orig.*, 1923, xxxvii, 106.

[12] Dilution was resorted to in order to shorten the subsequent titration series.

[13] Appelmans, R., *Compt. rend. Soc. biol.*, 1921, lxxxv, 1098.

[14] Werthemann, A., *Arch. Hyg.*, 1922, xci, 255.

[15] In the case of the extremely acid (pH = 2) and extremely alkaline (pH = 12) mixtures, the addition of 0.1 cc. of these mixtures to the 9.9 cc. of broth caused a drop or rise of the pH of the medium of less than 0.2.

perature (+7°C.). However, we carried out one of the experiments at both +7°C. and 37°C., in order to ascertain the effect of higher temperature on the rate of deterioration of the lytic principle at different pH. In the experiment mentioned lytic filtrate was allowed to remain in contact with various buffers, at 7°C., and at 37°C., for 3, 6, and 24 hours respectively. At the end of each period a sample (0.1 cc.) was taken from each mixture and titrated by serial dilution.[13, 14] The results of the titrations were first read at the end of 24 hours incubation at 37°C.; then again at the end of a further period of 24 hours of incubation. At this time the lytic titer in each of the tubes which received originally one or more active lytic units would have reached its maximum. For this reason, the tubes in which the occurrence of lysis was doubtful were heated for 30 minutes at 56°C., in order to destroy the resistant bacteria, and now 0.1 cc. of the contents of such tubes was transferred into a fresh tube containing 10 cc. of sterile broth, seeded with 0.1 cc. of an emulsion containing 1,000,000,000 susceptible bacteria per cc., and incubated for 3 to 4 hours at 37°C. At the end of this time, and before any overgrowth had taken place, the final readings were made.

As an illustration, results of the experiment at 7°C. are shown in Protocol 1. The sign + in this protocol signifies the presence of lysis, as judged by absence or slight amount of visible growth, and the sign − signifies apparent absence of lysis, or presence of bacterial growth equal or nearly equal to that occurring in a control tube of broth receiving 0.1 cc. of bacterial suspension only.

It will be seen from this protocol that direct readings of the results of titration, after 24 or 48 hours, were somewhat confusing, owing to the fact that the overgrowth of resistant bacteria often masks the coincident lysis of susceptible individuals in the culture. It is for this reason that a final test, by means of a transfer of 0.1 cc. from each tube (after heating) was resorted to, as stated above.

The results recorded in the protocol represent quantitatively the changes in activity of the Laudman Shiga lytic filtrate, which took place after its exposure to various buffers for 3 hours at + 7°C. Identical titrations were performed after a period of 6 and 24 hours contact of filtrate with buffers at 7°C., as well as after a contact for the same periods of time at 37°C. The results of all these titrations are presented graphically on Chart 1, in terms of the minimum amount of the original mixture (Protocol 1, *A*) which contained at least one active unit of the lytic principle after the exposure to buffers for a fixed length of time, at a fixed temperature.

Protocol 1.

Effect of the Hydrogen Ion Concentration on Activity of the Lytic Filtrate Laudman Shiga at +7°C.

A	Laudman Shiga filtrate diluted 1:1000, *cc.*	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
	Buffer solutions, *cc.*	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	1.8	
	pH of buffer solutions (approximate)	2.0	3.0	4.0	5.0	6.0	7.4	8.6	9.8	11.0	12.0	
	Resulting pH of mixture (electrometric)	2.05	3.1	4.1	5.1	6.15	7.4	8.77	9.75	11.25	12.07	
	Mixed. Left in ice box. Samples taken after 3 hrs.											
	Amount of sample, *cc.*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.0
	Buffered (pH = 7.4) broth, *cc.*	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	9.9	10.0
	Resulting pH (electrometric)	7.25					7.4				7.6	7.4

1 cc. of the contents of each tube, after thorough mixing, transferred into 9.0 cc. of broth, and 1 cc. from that tube into 9.0 cc. of broth in the next series, and so on.

	Absolute amount of the original lytic filtrate present.	Amount of Mixture A present.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.	24 hrs.	48 hrs.	Final transfer.
	cc.	*cc.*																																							
Results of titration after 3 hrs.	1×10^{-5}	0.1	−	−	−	−	−	−	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	−	−	+	+	+	+	−	−	−								
	1×10^{-6}	0.01	−	−	−	−	−	−	+	−	+	−	−	+	+	−	+	−	−	+	−	−	+	−	−	+	−	−	−	−	−	−								
	1×10^{-7}	0.001	−	−	−	−	−	−	+	±	+	+	±	+	+	±	+	+	±	+	−	−	+	+	−	+	−	−	−	−	−	−								
	1×10^{-8}	0.0001	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	±	−	+	+	−	+	−	−	−	−	−	−								
	1×10^{-9}	0.00001	−	−	−	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	−	+	+	−	+	−	−	−	−	−	−								
	1×10^{-10}	0.000001	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	+*	−	−	−	−	−	−								
	1×10^{-11}	0.0000001	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−	−								
	Control.	0.0																														−	−	−							

* Occasional activity in this dilution was due to particulate nature of distribution of the active principle (see earlier paper: Bronfenbrenner, J. J., and Korb, C., *J. Exp. Med.*, 1925, xlii, 483).

Comparative Resistance to Changes in Hydrogen Ion Concentration.

As can be seen from the chart, the destruction of the lytic power of the filtrate is considerably greater at 37°C. than at 7°C. So far as the effect of the length of exposure is concerned, in either case practically the entire change takes place very early, so that very little fur-

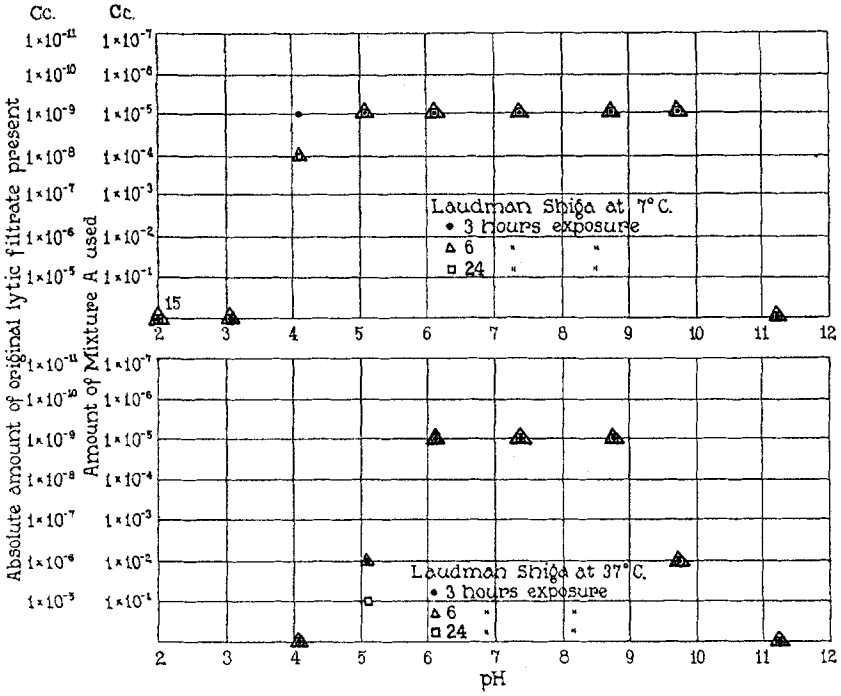

CHART 1. Effect of changes in the hydrogen ion concentration on the activity of lytic filtrate. On this as well as on the two other charts the points plotted on the base line indicate that there was no lysis in the largest amounts used (1×10^{-1}).

ther deterioration can be noted after the lapse of the first 3 hours. For the reasons already stated, in all further experiments the exposure of lytic filtrates to different buffers was carried out at 7°C. only, and, because of the above findings, the exposure in all cases was limited to 3 hours.

Using the procedure described, we have studied the resistance to changes in the hydrogen ion concentration of lytic filtrates active

respectively against *Bacillus coli*, *Bacillus dysenteriæ* Shiga, *Bacillus pestis caviæ*, and staphylococcus. Since the protocols of these experiments are in every detail similar to Protocol 1, further protocols have been omitted, and the findings have been recorded in Chart 2, in the manner identical with that used for construction of Chart 1.

Effect of "Adaptation" on the Characteristic Resistances.

The data plotted on Chart 2 have been obtained repeatedly, thus indicating that the various lytic agents studied have individual curves

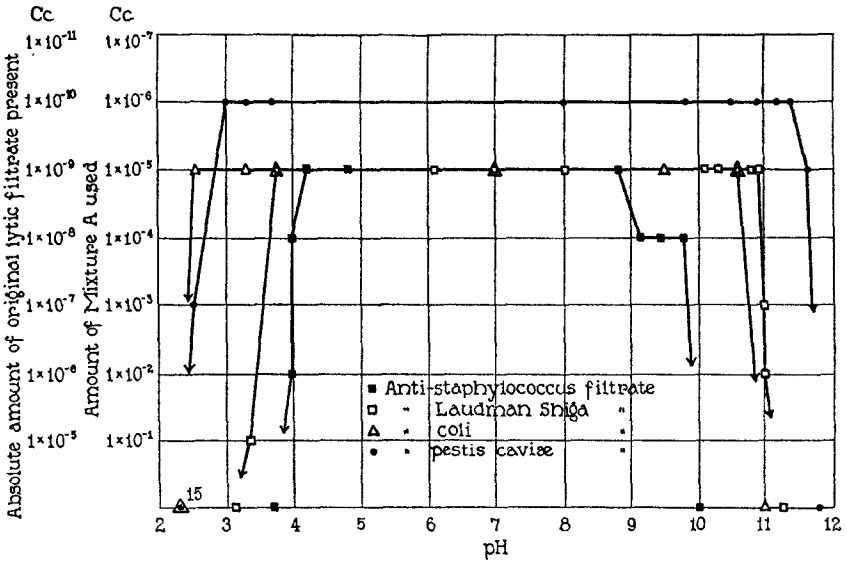

CHART 2. Comparative resistance of different lytic filtrates to changes in hydrogen ion concentration.

of resistance to changes in hydrogen ion concentration. The lytic agents active against *Bacillus pestis caviæ* seem to be the most resistant, remaining unaffected in the wide zone between pH = 3 and pH = 11.45. The lytic agent active against staphylococcus, on the other hand, is the least resistant, its resistance being limited by the hydrogen ion concentration corresponding to pH = 4.2 on the acid side, and pH = 8.8 on the alkaline side. The lytic filtrates active against *Bacillus coli* and *Bacillus dysenteriæ* Shiga resist alkalinity in almost the same degree, the limiting alkalinity for the former being pH = 10.6 and for

the latter pH = 10.9. On the acid side the lytic agent active against *Bacillus coli* is limited by pH = 2.55, whereas that active against *Bacillus dysenteriæ* Shiga deteriorates beyond pH = 3.75.

If one accepts d'Hérelle's conception that there is only one lytic principle these differences would have to be explained as resulting from "adaptation" of the virus to different "hosts."

In order to determine whether the change in bacterial species serving as substratum for the production of lytic filtrate is sufficient to cause the change in its resistance to acidity or alkalinity, the following experiment was undertaken.

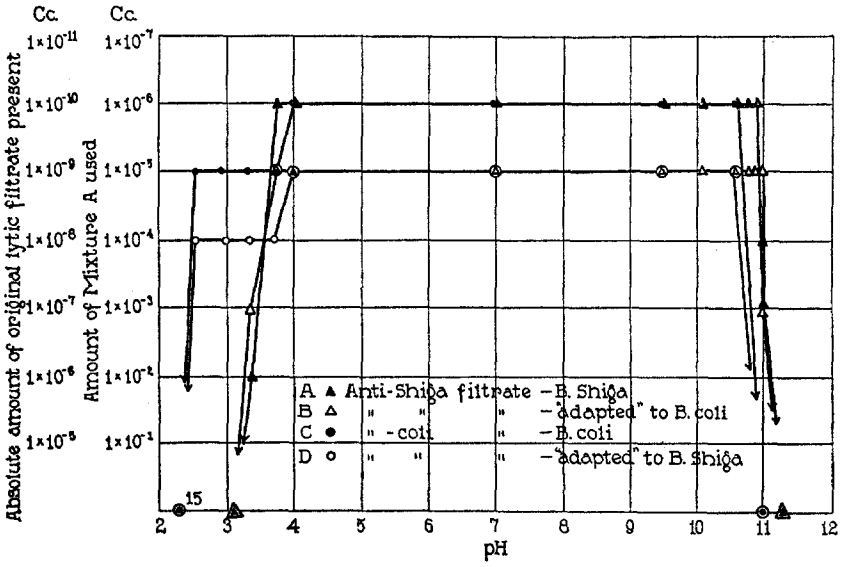

CHART 3. Effect of "adaptation" on resistance of lytic filtrates to changes in hydrogen ion concentration.

Starting with a polyvalent lytic principle, acting primarily against *B. dysenteriæ* Shiga and secondarily against *B. coli*, two lytic filtrates were prepared; one (*A*), by passage through a number of generations upon a culture of *B. dysenteriæ* Shiga, and the other (*B*) upon a culture of *B. coli*. The number of passages (ten) was amply sufficient to have eliminated all traces of activity due to the original filtrate. At the end of these passages the filtrates thus obtained caused lysis of the respective bacteria when present in culture in the amounts of 10^{-10} and 10^{-9} cc. respectively.

In a similar manner two filtrates were prepared, starting with another polyvalent

bacteriophage, active principally against *B. coli*, though causing also a less complete lysis of Shiga bacillus. In this case one filtrate represented the result of many passages on a culture of *B. coli* (*C*), and the other ten passages on Shiga bacillus (*D*).

All four filtrates were diluted with buffer solutions at various pH and titrated after exposure to them for 3 hours at 7°C., exactly as before. The results of these titrations have been plotted on Chart 3.

Although the initial titers of both the filtrates of the "adapted" bacteriophages (*B* and *D*) were somewhat lower than those of the respective original ones (*A* and *C*), nevertheless the resistance to changes in hydrogen ion concentration was identical for each original and its adapted material.

SUMMARY AND CONCLUSIONS.

Lytic filtrates, active against *Bacillus dysenteriæ* Shiga, *Bacillus coli*, *Bacillus pestis caviæ*, and staphylococcus respectively, proved to be differently affected by changes in hydrogen ion concentration.

Anti-staphylococcus lysin was the least resistant of the four, showing deterioration in 3 hours at 7°C. beyond the zone of hydrogen ion concentration limited by $C_H = 6.3 \times 10^{-5}$ and $C_H = 1.6 \times 10^{-9}$. Under the same conditions, the zone of resistance of anti-*coli* filtrate lay between $C_H = 2.7 \times 10^{-3}$ and $C_H = 2.5 \times 10^{-11}$, and that of anti-Shiga between $C_H = 1.7 \times 10^{-4}$ and $C_H = 1.3 \times 10^{-11}$. Anti-*pestis caviæ* filtrate was most resistant of the four, retaining its full activity in the zone from $C_H = 1 \times 10^{-3}$ to $C_H = 3.5 \times 10^{-12}$.

The fact that these differences in individual resistance persisted, notwithstanding the repeated passage of lytic filtrates through cultures of bacteria other than those against which they were primarily active, seems to offer evidence in favor of a multiplicity of bacteriophages.