STUDIES ON THE BACTERIOPHAGE OF D'HÉRELLE.

II. Effect of Alcohol on the Bacteriophage of d'Hérelle.

By JACQUES J. BRONFENBRENNER, Ph.D., and CHARLES KORB, M.D.

(From the Laboratories of The Rockefeller Institute for Medical Research.)

(Received for publication, June 6, 1925.)

In the course of some experiments in which we had occasion to precipitate lytic filtrates with alcohol, we observed that in many instances such precipitates manifested appreciable lytic action. Because of the divergent statements in the literature upon this point and the interest which the effect of alcohol on the lytic principle might have in connection with the question of the animate nature of the bacteriophage, we have attempted a brief inquiry into this question.

D'Hérelle[1] has reported that by precipitating a filtrate of antidysentery bacteriophage with 9 volumes of 96 per cent alcohol, decanting the supernatant fluid after 48 hours, and redissolving the precipitate in saline, he obtained a substance slightly lytic for dysentery bacilli. This lytic action, however, was not transmissible in series. D'Hérelle concluded that the alcohol destroyed the living ultramicrobe (bacteriophage) and precipitated its endoenzyme which was responsible for lysis in the first generation. Hauduroy,[2] repeating the experiment of d'Hérelle, obtained a similar result by precipitating sterile bouillon with alcohol. He suggested, therefore, that the apparent lytic action of the precipitate obtained by d'Hérelle was due to the bacteriostatic effect of the alcohol adsorbed by this precipitate. Complete destruction of the lytic activity of bacteriophage by alcohol was also observed by Watanabe[3] and by Arnold.[4]

On the other hand, in the course of some other work, Kabéshima[5] states that he has separated bacteriophage from its solution by precipitation with alcohol. This author, however, does not indicate the concentration of alcohol used nor the

[1] d'Hérelle, F., Le bactériophage. Son rôle dans l'immunité, Monographies de l' Institut Pasteur, Paris, 1921, 95.

[2] Hauduroy, P., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 964.

[3] Watanabe, T., *Arch. Hyg.*, 1923–24, xcii, 1.

[4] Arnold, L., *J. Lab. and Clin. Med.*, 1922–23, viii, 720.

[5] Kabéshima, T., *Compt. rend. Soc. biol.*, 1920, lxxxiii, 219.

length of exposure. Appelmans,[6] too, found that bacteriophage action is not completely destroyed even after 20 days exposure to alcohol in 50 per cent concentration. He does not state whether the lytic action observed by him is transmissible in series, or, like that observed by d'Hérelle, is demonstrable in first generation only.

Our experiments were first made with two strains of bacteriophage active against *Bacillus dysenteriæ* Shiga and Flexner respectively.[7] Later, similar results were obtained with a bacteriophage active against colon bacillus and with two strains of bacteriophage active against *Bacillus enteritidis*, M. T. I; and *Bacillus pestis caviæ* M. T. II respectively.[8]

The general procedure adopted in the experiments was as follows:

Several portions of 1 cc. each of active filtrate were carefully deposited at the bottom of sterile centrifuge tubes by means of a pipette in such a way as to exclude any contact with the walls of the upper portion of the tubes. Into each tube was then introduced 9 cc. of 95 per cent alcohol, and after thorough mixing of the contents, the mixtures were overlaid carefully with 1 cc. more of 95 per cent alcohol to exclude the possibility of deposition of droplets of the lytic principle on the walls of the tube above the surface of the alcohol. The tubes were kept at room temperature and at stated intervals the contents were centrifuged for 10 minutes, the liquid decanted, and the precipitate taken up in 1 cc. of sterile physiological salt solution. Both the alcohol fraction and the redissolved precipitate were titrated for their respective lytic power by the tenfold dilution method of Appelmans[6] as modified by Werthemann.[9] All dilutions for titration were made in broth adjusted to a slightly alkaline reaction (pH = 7.4) and containing about 1,000,000 young susceptible bacteria in each cc. A fresh sterile pipette was used for each dilution. The presence of active principle in the tubes exhibiting a doubtful degree of lysis or a possible overgrowth of resistant bacteria was at first controlled in the usual manner—by transfer to agar slants previously seeded with susceptible bacteria. Later it was found more satisfactory to incubate such tubes for 36 to 48 hours, thus allowing the lytic titer to reach its maximum in all the tubes where there was any bacteriophage present. At the end of this time the

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

[7] These strains of bacteriophage were isolated by us from stools obtained from the Babies' Hospital through the kind cooperation of Dr. Martha Wollstein.

[8] The last two strains of bacteriophage were selected on account of their high specificity among seven strains isolated by us from stools of mice surviving the experimental infection and kindly placed at our disposal by Dr. Leslie T. Webster and Dr. Ida W. Pritchett.

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

Protocol 1.

The Effect of Alcohol on the Lytic Activity of Bacteriophage at Room Temperature

Laudman Shiga bacteriophage.....	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.	1.0 cc.
95 per cent alcohol.....	0.0 " (control).	10.0 "	10.0 "	10.0 "	10.0 "	10.0 "

Kept at room temperature for periods indicated below; centrifuged for 10 min. after time as stated; precipitate taken up in 1 cc. 0.85 per cent salt solution; alcohol-soluble fraction (supernatant fluid) and the precipitated fraction titrated separately.

Length of exposure to alcohol.....				10 min.				1¼ hrs.				3 hrs.				6 hrs.				24 hrs.			
				Supernatant.			Precipitate.	Supernatant.			Precipitate.	Supernatant.			Precipitate.	Supernatant.			Precipitate.	Supernatant.			Precipitate.
	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.	Lysis in broth.	Agar transfer.	Serial lysis.
cc.																										
10^{-1}	+			+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
10^{-2}	+			+	+	+	+	−	−	−	+	+	+	−	+	+	+	−	−	−	+	+	+	−	−	−
10^{-3}	+			+	+	+	+	−	−		+	+	+	−	+	+	+	−	−		+	+	+	−	−	
10^{-4}	+			−	−	−	+	−	−		+	+	+		+	+	+	−	−		−	−	−	−	−	
10^{-5}	+			−	−		+	−	−		−	−	−		−	−	−	−	−		−	−	−	−	−	
10^{-6}	+			−	−		−	−	−		−	−	−		−	−	−	−	−		−	−	−	−	−	
10^{-7}	+			−	−			−			−	−			−	−		−			−	−		−	−	
10^{-8}	+	+	+	−				−																		
10^{-9}	+	+	+																							
10^{-10}	+	+	+																							
10^{-11}	−	−	−																							

+ = lysis of bacteria; − = no lysis.

tubes were heated for 30 minutes at 56°C. and their contents were tested for lytic activity by transferring 0.1 cc. from each of these tubes into broth seeded with susceptible bacteria. In all cases where bacteriophage was present the lysis took place in 3 to 4 hours and was transmissible in series.

The protocol of one of these experiments (Protocol 1) is given above. With slight modifications, which will be indicated, all the experiments followed the general scheme outlined in this protocol. The repetition of the protocol will therefore be omitted, and the results will be tabulated in terms of minimum amount of solution exhibiting lytic activity. The results shown in Protocol 1 are tabulated in Table I.

TABLE I.

Inactivation of Bacteriophage by Alcohol at Room Temperature.

1 cc. Laudman Shiga bacteriophage + 10 cc. 95 per cent alcohol at room temperature.

Exposure to alcohol for.....	10 min.	1¼ hrs.	3 hrs.	6 hrs.	24 hrs.	
Lytic titer of supernatant alcohol.....	10^{-3} cc.	Not active in 10^{-2} cc.	Not active in 10^{-2} cc.	Not active in 10^{-2} cc.	Not active in* 10^{-2} cc.	
Lytic titer of precipitate....	10^{-5} cc.	10^{-3} cc.	10^{-3} cc.	10^{-2} cc.	Not active in 10^{-1} cc.	
Original lytic titer.....						10^{-10} cc.

* The supernatant alcohol was not tested in amounts above 0.1 cc. corresponding to 0.01 cc. (or 10^{-2} cc.) of the original filtrate to avoid bacteriostatic effect of alcohol.

From Table I it is seen that only a trace of lytic principle was demonstrable in the supernatant fluid after the first 10 minutes of contact with alcohol, while in the precipitate measurable activity persisted for over 6 hours. Moreover, as long as this lytic activity persisted, it was consistently transmissible in series. We were unable to observe the non-transmissible lysis ascribed by d'Hérelle[1] to the action of an endoenzyme. Thus it appears that the bacteriophages we employed are completely inactivated by contact with 10 volumes of 95 per cent alcohol at room temperature in less than 24 hours.

D'Hérelle lays great stress on this susceptibility of bacteriophage to the action of alcohol as one of the important links in the chain of evidence in favor of its animate nature. While it is true that if the bacteriophage were a living parasite one would expect it to be affected by alcohol, as is the case with most if not all living organisms,[10] yet the converse does not necessarily follow. The inactivation of bacteriophage by alcohol does not necessarily indicate its living nature since alcohol may conceivably denature an inanimate organic substance sufficiently to modify or destroy some of its properties. Certain enzymes, for instance, are very quickly inactivated by the alcohol.[11] Alcohol also destroys the potency of bacterial toxins in general, and of *botulinus* toxin in particular.[12] While this inactivation is most rapid at higher temperatures, we observed that even at 5°C. the potency of *botulinus* toxin is reduced 30,000 times in 10 minutes.[13]

Suspecting that the action of alcohol on bacteriophage may have the character of such a denaturation, we thought that perhaps at a lower temperature this denaturation would be sufficiently delayed to permit a close study of the process.

With this in view, the above experiment was repeated in every detail except that both the lytic filtrate and the alcohol were cooled, prior to mixing, and were left, after mixing, at the same temperature (6–7°C.) for different periods of time, as indicated in Table II.

At stated intervals the mixtures were centrifuged, the supernatant fluid was separated from the precipitate, and both were titrated by serial dilution as before. It will be seen from Chart 1 by comparing the results recorded in Table II with those of the preceding experiment (Table I) that, whereas at room temperature complete inactivation of lytic power of the filtrates by alcohol required only about 1 hour for the supernatant fluid and more than 6 hours for the precipitable fraction, at ice box temperature the supernatant fluid exhibited

[10] Rous sarcoma virus according to Funk, as well as virus of mosaic disease of plants according to Iwanowski (Iwanowski, D., *Centr. Bakt., 2. Abt.*, 1899, v, 250), is not affected by alcohol.

[11] Hudson, C. S., and Paine, H. S., *J. Am. Chem. Soc.*, 1910, xxxii, 1350.

[12] Bronfenbrenner, J., and Schlesinger, M. J., *Proc. Soc. Exp. Biol. and Med.*, 1920–21, xviii, 304.

[13] Bronfenbrenner, J., unpublished experiments.

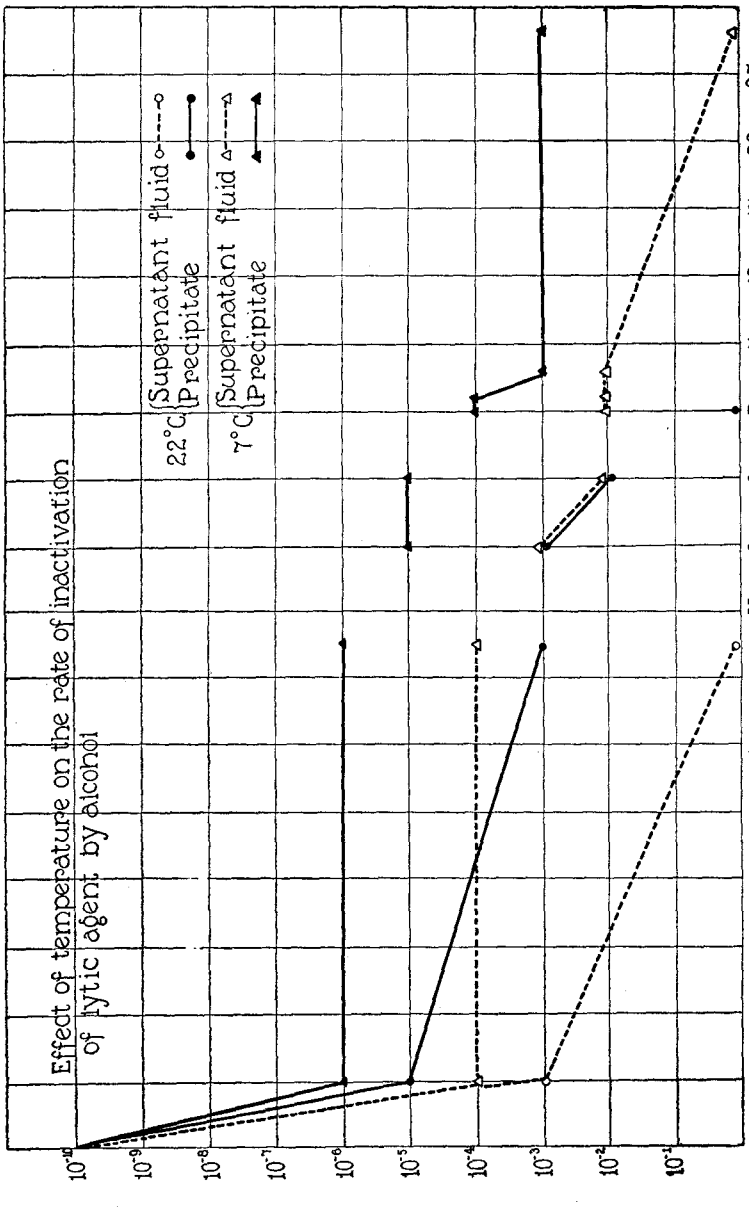
Effect of temperature on the rate of inactivation of lytic agent by alcohol
22°C (Supernatant fluid
Precipitate
7°C (Supernatant fluid
Precipitate
Min. 0 10 20 30 40 50 60 70 Hrs. 3 6 10 15 20 25 Days
10^{-10} 10^{-9} 10^{-8} 10^{-7} 10^{-6} 10^{-5} 10^{-4} 10^{-3} 10^{-2} 10^{-1}

CHART 1.

some degree of activity even after 4 days, and the precipitate still caused serial lysis after the exposure to ten times its volume of 95 per cent alcohol for 4 weeks.

These experiments were repeated several times and with a number of different lytic filtrates with similar results, notably at ice box temperature. At room temperature the time for complete loss of lytic activity of the precipitate varied with different filtrates from 3 to 24 hours.

While the final inactivation of the lytic filtrates by the alcohol was slower at ice box temperature, it is to be noted that almost the same marked initial reduction of lytic activity took place at ice box as at

TABLE II.

Inactivation of Bacteriophage by Alcohol at 7°C.

1 cc. Laudman Shiga bacteriophage + 10 cc. 95 per cent alcohol at ice box temperature (+7°C.).

Exposure to alcohol for.....	10 min.	1¼ hrs.	3 hrs.	6 hrs.	24 hrs.	44 hrs.	4 days.	28 days.	
Lytic titer of supernatant alcohol.....	10^{-4} cc.	10^{-4} cc.	10^{-3} cc.	10^{-2} cc.	10^{-2} cc.	10^{-2} cc.	10^{-2} cc.	Not active in 1×10^{-2} cc.	
Lytic titer of precipitate.....	10^{-6} cc.	10^{-6} cc.	10^{-5} cc.	10^{-5} cc.	10^{-4} cc.	10^{-4} cc.	10^{-3} cc.	10^{-3} cc.	
Original lytic titer....									10^{-10} cc.

room temperature.[14] Since the tubes, in order to bring about titration of their contents, had to be taken into the room to be balanced preliminary to centrifuging, and then centrifuged, it was thought that in these 15 to 20 minutes the change in the temperature of the mixtures might be sufficient to account for the rapid initial inactivation almost equal to that occurring when the entire process was carried out at room temperature.

In order to prevent this rise in temperature of the cooled bacteriophage-alcohol mixtures during centrifuging, this latter step was

[14] Bronfenbrenner, J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 177.

omitted. After varying intervals the precipitate which appeared following the addition of alcohol to the lytic filtrate was thoroughly suspended by repeated aspiration into a pipette. From the resulting homogeneous suspension, 0.1 cc. was transferred each time directly into broth for titration by further dilution in series, as in the previous tests.

The results of this titration indicate that when the temperature was kept at 7°C. throughout the experiment, the inactivation of the filtrate by the alcohol was appreciably slower than at room temperature. However, even under these circumstances, lytic activity of the filtrate was reduced to 1 per cent of its original value within 1 hour after the exposure to alcohol (Table III).

TABLE III.

Combined Lytic Activity of the Supernatant Fluid and of Sediment after Exposure to Alcohol at 7°C.

1 cc. Laudman Shiga bacteriophage + 10 cc. 95 per cent alcohol at 7°C.

Exposure to alcohol for..	15 min.	1 hr.	3 hrs.	4½ hrs.	7 hrs.	24 hrs.	48 hrs.	4 days.	12 days.	24 days.	
Lytic titer of the mixture.....	10^{-9} cc.	10^{-8} cc.	10^{-7} cc.	10^{-7} cc.	10^{-6} cc.	10^{-5} cc.	10^{-4} cc.	10^{-3} cc.	10^{-3} cc.	10^{-3} cc.	
Original titer of the phage.....											10^{-10} cc.

Rate of Destruction of Bacteriophage on Second Precipitation by Alcohol.

While the loss of lytic activity in the preceding experiment was equal to 99 per cent in the 1st hour after exposure to alcohol (from lytic titer of 10^{-10} cc. to 10^{-8} cc.), further reduction of activity seemed to take place at a progressively slower rate.

Since it has been suggested in the literature[15,16] that lytic filtrates may consist of several component fractions differing in their respective

[15] Kouo-Ngen, J. T., and Wagemans, J., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 1253; 1923, lxxxviii, 303.

[16] Reichert, F., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 235.

resistance to injurious agents, the possibility that the greater initial rate of destruction of bacteriophage by the alcohol may be due to the presence of a certain fraction which is particularly susceptible to the action of alcohol was inquired into.

Two portions of cooled lytic filtrate, of 1 cc. each, were placed in two centrifuge tubes, precipitated with 10 cc. of cooled alcohol, and placed at 7°C. After stated periods the contents of each tube was thoroughly mixed and 0.1 cc. samples were taken out for titration to determine the rate of inactivation of the lysin. After 20

Protocol 2.

Inactivation of Bacteriophage by Repeated Treatment with Alcohol at 7°C.

	cc.	cc.	cc.
Cooled filtrate	1	1	1
Cooled 95 per cent alcohol	10	10	—
Physiological salt solution	—	—	10
Placed at 7°C. and titrated at intervals.			
Initial lytic titer			10^{-10}
Lytic titer after 2 hrs.	10^{-8}	10^{-8}	
" " " 4 "	10^{-7}	10^{-7}	
" " " 20 "	10^{-6}	10^{-6}	
Centrifuged at 10°C. Precipitate taken up in 1 cc. of cooled salt solution.			
Lytic titer (20 hrs.)	10^{-6}		
Cooled 95 per cent alcohol	10		
Placed at 7°C.			
Lytic titer after 24 hrs.	10^{-3}	10^{-5}	
" " " 42 "	10^{-2}	10^{-4}	10^{-10}

hours of contact with alcohol, one of the tubes was subjected to centrifuging (in a specially cooled centrifuge). The precipitate thus obtained was separated from the supernatant alcohol and taken up in 1 cc. of cooled physiological salt solution. A sample (0.1 cc.) of this solution was transferred into broth for immediate titration to determine the extent of its activity at that time, and the remaining solution was again precipitated by 10 volumes of 95 per cent alcohol, and replaced in the ice box. At intervals the contents of this tube, as well as those of the second tube (which remained undisturbed in the ice box, as control), were thoroughly mixed and samples taken for titration by serial dilution.

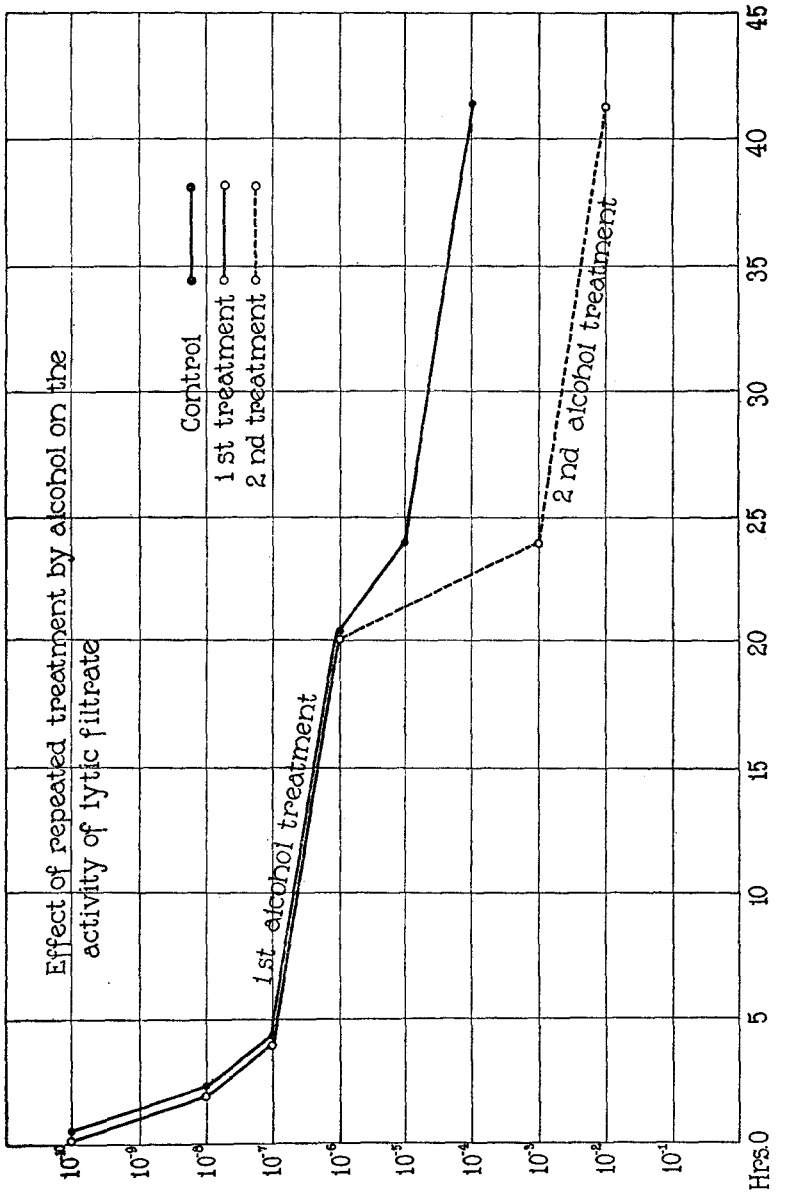
Effect of repeated treatment by alcohol on the activity of lytic filtrate
Control
1 st treatment
2 nd treatment
1st alcohol treatment
2 nd alcohol treatment
10⁻⁸
10⁻⁹
10⁻⁸
10⁻⁷
10⁻⁶
10⁻⁵
10⁻⁴
10⁻³
10⁻²
10⁻¹
Hrs.0
5
10
15
20
25
30
35
40
45

CHART 2.

The results of these titrations are plotted in Chart 2 from the data presented in Protocol 2. As can be seen the inactivation of lysin in the first period of the experiment went on in the same way in both tubes. During the first 2 hours of contact with alcohol there was a one hundredfold diminution in the activity of the lysin which further decreased tenfold in the 2 hours following. When, 20 hours later, the remaining lysin was again subjected to the action of alcohol, the rate of its deterioration duplicated that observed in the initial stages of the first precipitation with alcohol.

It appears, therefore, that the susceptibility of the lytic principle to alcohol is uniform throughout, and that the observed difference in the rate of deterioration at the later stages of exposure is not due to the presence of a more resistant fraction.

SUMMARY AND CONCLUSIONS.

When bacteriophage is precipitated by alcohol at room temperature its activity rapidly and progressively decreases until it is totally destroyed, between 6 and 24 hours after exposure.

If the percipitation is carried out at 7°C. the destruction of lytic activity is considerably slower; measurable traces may be detected even after 4 weeks exposure to alcohol. Although the major portion of the lytic activity is found in the precipitate, the supernatant alcohol carries a measurable amount of lytic principle which remains active for several days.

In all cases the residual lytic activity was found to be transmissible in series. In no instance were we able to observe the non-transmissible action ascribed by d'Hérelle to the enzyme.

The persistence of traces of active principle after many weeks of exposure to alcohol at low temperature is not found to be due to the existence in the original filtrate of a fraction relatively resistant to the effect of alcohol.

The inactivation of bacteriophage by alcohol seems, therefore, analogous to the alcoholic inactivation of certain enzymes and toxins.