STUDIES ON THE BACTERIOPHAGE OF D'HERELLE.

III. Some of the Factors Determining the Number and Size of Plaques of Bacterial Lysis on Agar.

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Plate 20.

(Received for publication, June 13, 1925.)

The quantitative evaluation of the activity of the so called bacteriophage is usually accomplished by one of two methods.

The earlier procedure and its modifications[1,2] make use of the appearance of clear spots—"*taches vierges*" or "plaques"—in he midst of an even growth when bacteria are seeded on agar in the presence of bacteriophage-containing solutions. It is assumed that each clear spot represents a colony of "bacteriophage," and the count of these colonies is carried out in a manner analogous to that used in counting colonies of bacteria.

The other method [3,4] consists in preliminary serial dilution of the bacteriophage-containing fluid in sterile broth, with subsequent inoculation of these serial dilutions with a standard suspension of susceptible bacteria. In this case it is assumed that the highest dilution in which the lysis of bacteria occurs after a proper incubation must have received at least one unit of lytic principle and thus the total number of active units present in a given volume of the original fluid may be computed.

If in the case of "bacteriophage" one is dealing with a suspension of discrete living elements, as assumed by d'Hérelle, the count of these particles by both methods should be consistent, as it is when used in enumerating bacteria, and therefore the methods could be used inter-

[1] d'Hérelle, F., Le bactériophage, son rôle dans l'immunité, Monographies de l'Institut Pasteur, Paris, 1921, 17.

[2] Gildemeister, E., and Herzberg, K., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 12.

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

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

changeably. However, the observations of several investigators,[5-8] as well as our own experience,[9] have shown that the lytic titers of bacteriophage-containing fluids are considerably higher when determined by the broth dilution method than on counting the plaques on agar. Before going into the reason for this discrepancy, we have felt that it is necessary to establish first whether the lytic principle is really particulate, as assumed by d'Hérelle, or whether, on the contrary, it is present in a state of true solution.

Particulate Distribution of the Lytic Principle.

When a broth culture of young, susceptible bacteria is allowed to grow in the presence of suitable amounts of the active lytic principle, the concentration of the latter can be shown to increase rapidly during the period of logarithmic growth of bacteria. After 18 to 24 hours of incubation the concentration of the lytic principle usually reaches its highest level, after which it remains unchanged, irrespective of further changes in the bacterial count. If at the time bacteria are removed by filtration, the filtrate is distributed in a series of broth tubes in gradually diminishing amounts, from 0.1 cc. down, and if each of the tubes of the series is seeded with 0.1 cc. of a suspension of young culture (18 hours) containing about 1,000,000,000 of susceptible bacteria per cc., one finds that after a suitable period of time (18 to 24 hours) lysis of bacteria has taken place in all tubes receiving more than 10^{-10} cc. of the filtrate. All the tubes receiving less than 10^{-10} cc., with but few exceptions, show no lysis. Moreover, if the tubes are incubated further for 18 to 24 hours, and if at this time their contents are heated, or filtered, to kill, or remove, the secondary growth of resistant bacteria, and titrated for lytic activity by the serial dilution method,[3,4] one finds that the concentration of the lytic principle is at the same maximum in all the tubes in which lysis was observed

[5] Doerr, R., *Schweiz. med. Woch.*, 1923, iv, 1009.

[6] Doerr, R., and Rose, G., *Schweiz. med. Woch.*, 1924, v, 10.

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

[8] Gildemeister, E., and Herzberg, K., *Centr. Bakt., 1. Abt., Orig.*, 1923–24, xci, 228.

[9] Bronfenbrenner, J., and Korb, C., *Proc. Soc. Exp. Biol. and Med.*, 1923–24, xxi, 315.

earlier, irrespective of the amount of lytic filtrate they received originally. On the other hand, all the tubes receiving less than 10^{-10} cc. of the filtrate show no lytic activity.

The results of the experiment illustrated in Protocol 1 suggest that the lytic agent is present in the filtrate not in the form of true solution, but in a particulate state. Thus, the fact that 10^{-10} cc. of the filtrate produces lysis, whereas 10^{-11} cc. produces no lysis is explained as showing that 10^{-10} cc. contains at least one and less than ten indivisible active particles of bacteriophage. Since the distribution of such discrete particles in very high dilutions may be such that not every cc. of dilution will contain a particle, it is scarcely surprising that in the next dilution (1×10^{-11} cc.) we find two tubes only (Nos. 2 and 8) showing lytic activity.

If, then, it is true that 1×10^{-10} cc. contains one indivisible particle of the active principle, it should follow that one-half or one-third of this amount will contain less than one, and hence show no lytic action. If, however, the lytic agent is present in the form of a true solution, combination of three portions of $1/3 \times 10^{-10}$ cc. should result in the same activity as is produced by one whole portion of 1×10^{-10} cc. Or, the lytic agent may be present in a measurable amount in even 1×10^{-11} cc., which concentration, however, would appear too low to induce a change in bacteria resulting in their lysis. In this event a combination of 10 portions of 1×10^{-11} cc. should be active. Should this prove to be the case, the true solubility, as opposed to particulate distribution of bacteriophage, would be established. The following experiment is one of a series of similar ones carried out to obtain information on this point.

Active filtrate Laudman Shiga was diluted with sterile broth so that each cc. of dilution contained 1×10^{-9} cc. of the original filtrate. 2 cc. of this dilution was added to 198 cc. of sterile broth, mixed thoroughly, and distributed in 10 cc. amounts in each of twenty sterile tubes. Thus each of the tubes of the series received 1×10^{-10} cc. of the original filtrate (Protocol 2, *A*). In a similar manner another series of twenty tubes was prepared to contain in each 1×10^{-11} cc. of the original filtrate (Protocol 2, *B*). Each of the tubes was then seeded with 0.1 cc. of a suspension of *Bacillus dysenteriæ* Shiga containing 10^9 susceptible bacteria per cc. and incubated at 37°C. The appearance of lysis was recorded at the end of 18 hours, as well as at the end of 40 hours of incubation. At this time all the tubes were heated to destroy the bacteria, and a drop of the contents was deposited on a

*Protocol 1.**

Increase of Lytic Principle to a Maximum.

Amount of active filtrate in 0.1 cc. volume, *cc.*	10^{-1}	10^{-2}	10^{-3}	10^{-4}	10^{-5}	10^{-6}	10^{-7}	10^{-8}	10^{-9}	10^{-10}	10^{-11}	10^{-12}	0
Broth, *cc.*	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.8	9.9
Bacterial suspension, *cc.*	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Incubated at 37° C. for 18 to 24 hrs.													
Results	+	+	+	+	+	+	±	±	±	±	−	−	−
Incubated again for 18 hrs. at 37°C., heated for 30 min. at 56°C. (or filtered), and titrated.													
Tube No.	1	2	3	4	5	6	7	8	9	10	11	12	13
Results of titration. 1×10^{-1} cc.	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-3} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-5} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-7} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-9} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-10} "	+	+	+	+	+	+	+	+	+	+	−	−	−
1×10^{-11} "	−	+	−	−	−	−	−	+	−	−	−	−	−
1×10^{-12} "	−	−	−	−	−	−	−	−	−	−	−	−	−

* In this as well as in following protocols the sign + signifies clearing of culture (or lysis of bacteria); the sign ± signifies a partial clearing; and the sign − signifies absence of lysis.

surface of agar, previously seeded with susceptible bacteria (agar transfer). This procedure served as a final check on the lytic activity of the contents of each tube.

Protocol 2.

Particulate Distribution of the Lytic Agent.

Series A.

2×10^{-9} cc. of lytic filtrate in 2 cc. volume + 198 cc. of sterile broth. Distributed in a series of twenty tubes, each receiving 1×10^{-10} cc. of filtrate in 10 cc. volume.

	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.
Tube No.....	1	2	3	4	5	6	7	8	9	10
Lysis.....	±++	±++	±++	±++	−−−	±++	±++	±++	−−−	±++
Tube No.....	11	12	13	14	15	16	17	18	19	20
Lysis.....	−−−	±++	±++	±++	±++	±++	±++	±++	−−−	±++

Contents of Tubes 5, 9, 11, and 19 were now combined, filtered, and the filtrate was seeded with susceptible bacteria. No lysis was observed.

Series B.

2×10^{-10}cc. of lytic filtrate in 2 cc. volume + 198 cc. of sterile broth. Distributed in a series of twenty tubes, each receiving 1×10^{-11} cc. of filtrate in 10 cc. volume.

	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.	18 hrs. 40 hrs. Agar transfer.
Tube No.....	1	2	3	4	5	6	7	8	9	10
Lysis.....	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	±++	−−−
Tube No.....	11	12	13	14	15	16	17	18	19	20
Lysis.....	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−	−−−

Contents of Tubes 1 to 8 and 10 to 20 were all combined, filtered, and the filtrate was seeded with susceptible bacteria, but no lysis could be observed.

As will be observed from the results presented in Protocol 2, a great majority of the tubes of Series A, each containing 10^{-10} cc. of the original filtrate, showed lysis, as did also a few of the tubes in Series B.

Protocol 3.

Effect of the Concentration of Agar on Lytic Activity.

5 per cent beef infusion agar, *cc.*	9	9	9	5	5	5									
2 per cent beef infusion agar, *cc.*							5	5	5	2.5	2.5	2.5	1.5	1.5	1.5
Beef infusion broth, *cc.*	0.0	0.0	0.0	4	4	4	4	4	4	6.5	6.5	6.5	7.5	7.5	7.5
B. dysenteriæ Shiga suspension, *cc.*	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9	0.9
0.1 cc. Duggan Shiga bacteriophage diluted in H_2O	1:10^4	1:10^6	1:10^8	1:10^4	1:10^6	1:10^8	1:10^7	1:10^8	1:10^9	1:10^7	1:10^8	1:10^9	1:10^7	1:10^8	1:10^9
Resulting agar concentration, *per cent*	4.5	4.5	4.5	2.5	2.5	2.5	1.0	1.0	1.0	0.5	0.5	0.5	0.3	0.3	0.3
Incubated at 37°C. overnight.															
Plaque count	0	0	0	15	0	0	8	1	0	39	5	0	0	75	7	0
Maximum diameter of plaques, *mm.*	—			0.25	—	—	1	—	—	2.5	—	—	4	—	—	
No. of plaques per cc. (titer).				($\pm$15x10^8) 0.00015 x 10^{10}			$\pm$0.1 x 10^{-10}			$\pm$0.5 x 10^{10}			$\pm$0.7 x 10^{10}			
No. of units per cc. as determined by broth titration																1 x 10^{10}

The contents of the tubes showing lysis were discarded and those showing absence of lytic activity were now combined, filtered, seeded with susceptible bacteria, and incubated at 37°C.

Since combination of the contents of several tubes containing insufficient amount of lytic principle in each does not result in bringing about lysis, it appears that the lytic principle is distributed through the medium in the state of indivisible units.

Effect of Concentration of Agar on Lytic Titer.

The results of the preceding experiment indicate that the lytic agent is distributed in the medium as if particulate. If these particles are living and multiplying individuals subject to numerical evaluation, as suggested by the early work, their count by dilution in broth should correspond with that obtained on agar. However, as stated above, this is not the case.

While investigating to explain this discrepancy, it occurred to us that the ease with which lytic principle is known to be adsorbed on a variety of colloidal substances [10–12] may be responsible for the lowering of lytic activity when tested on agar. It seemed logical to suppose that by decreasing the agar content of the medium one might cause the plate counts to approach more nearly the values obtained by parallel broth dilution titrations. Accordingly, experiments were performed in which anti-Shiga as well as anti-*coli* lytic filtrates were plated out on agar, varying in concentration from about 4.5 per cent to 0.25 per cent. In parallel titrations the lytic activity of these filtrates was also determined by the broth dilution method. Protocol 3 outlines one of many of these experiments.

The lytic filtrate Duggan Shiga used in this experiment when tested in broth caused lysis in the amount of 1×10^{-10} cc. Thus, as seen from Protocol 3, when the concentration of agar is reduced below 0.5 per cent, the count of plaques per cc. obtained thereon is 7×10^{9} or practically that obtained by the broth dilution method (1×10^{10}). On the other hand, when the concentration of agar is raised to 1 per

[10] Doerr, R., *Klin. Woch.*, 1922, i, 1489.

[11] Nakamura, O., *Arch. Hyg.*, 1923–24, xcii, 61.

[12] Doerr, R., and Berger, W., *Z. Hyg. u. Infectionskrankh.*, 1923, xcvii, 422.

cent, the readings are only 10 per cent of those obtained by broth titration, and if the concentration of agar is further increased, the titer becomes progressively lower.

Effect of Agar Concentration on the Plaque Size.

In addition to the changes in the number of plaques observed as the concentration of agar was varied, we found that in general the average size of plaques tended to increase as the concentration of agar

Protocol 4.

Effect of the Concentration of Agar on the Activity of Purified Lytic Principle.

Agar concentration, *per cent*.....	4.5	2.5	1	0.5
Amount of agar, *cc*.....	9	9	9	9
18 hr. culture of *B. dysenteriæ* Shiga, *cc*.....	0.9	0.9	0.9	0.9
0.1 cc. of Duggan Shiga filtrate diluted.....	$1:10^1$	$1:10^3$	$1:10^5$	$1:10^{10}$
Plaque count.....	0.0	130	180	281
No. of plaques per cc. (titer)	0.0	(130×10^4) 0.00013×10^{10}	(180×10^6) 0.018×10^{10}	(281×10^7) 0.28×10^{10}
Maximum diameter of plaques, *mm*.....	—	0.25	1	2.5

Plates from which the above figures were taken are shown in the photograph in slightly less than actual size (Figs. 1 to 4).

was reduced (Protocol 3). This phenomenon seemed to us of great interest since it suggests that the size of the plaques may be not only a function of a given lytic agent, as is usually believed, but it may also depend on the concentration of agar. Since the lytic agents used by us in these experiments were recently isolated by us from fecal material, they may conceivably have consisted of mixtures of different lytic agents which could account for the lack of constancy in the size of plaques.[13–18] We therefore purified these filtrates by repeated

[13] Gratia, A., *Compt. rend. Soc. biol.*, 1923, lxxxix, 821, 824.

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

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

[16] Wolff, L. K., and Janzen, J. W., *Ann. Inst. Pasteur*, 1923, xxxvii, 1064.

[17] Wagemans, J., *Arch. internat. pharmacod. et thérap.*, 1923–24, xxviii, 159, 181.

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

passages on agar, each time from a single plaque; when the lytic agents thus obtained appeared to give plaques of reasonable uniformity, we replated them on agar of varying concentrations (Protocol 4).

Similar changes in the size and number of plaques under the influence of variations in concentration of agar were observed with purified lytic filtrates (Phage B. W. and Pet. 2 respectively) sent to us by Gratia.[18]

When Irish moss was substituted for agar, results similar to those shown above were obtained. As the concentration of jelly was reduced the size and the number of plaques were increased.

Protocol 5.

Effect of an Excess of Susceptible Bacteria on the Size of Plaques.

Molten 2 per cent agar, *cc.*	9	9	9	9	9	9
B. dysenteriæ Shiga 18 hr. culture, No. of bacteria in 1 cc.	10^{10}	10^{9}	5×10^{8}	1×10^{8}	1×10^{7}	1×10^{6}
0.1 cc. Laudman Shiga filtrate diluted.	1:10^{6}	1:10^{6}	1:10^{6}	1:10^{6}	1:10^{6}	1:10^{6}
Plates poured and incubated at 37°C. overnight.						
Maximum size of plaques, *mm.*	0.25	0.5	0.5	0.75	Unsatisfactory discontinuous growth.	

Effect of the Number of Susceptible Bacteria on the Number and Size of Plaques.

Since it is known that bacteriophage principle does not multiply except in the presence of bacteria, and, moreover, since it is generally assumed that the development of plaques is the result of lysis of bacteria surrounding or developing on the spot in close proximity with the particles of active agent, it is possible that the number and size of such plaques will also depend on the density of bacterial suspension subjected to lysis. This relation was studied by pouring a series of agar plates in which, all other factors being constant, the total number of susceptible bacteria was varied.

As can be seen from the results presented in Protocol 5, when the number of bacteria added was less than 100 million per cc., the growth was discontinuous and the plaques were not clearly demarcated. As

the number of bacteria increased, the size of the plaques diminished, the total count of plaques not being appreciably affected.

Effect of Relative Concentration of Young and Old Bacteria.

It is known that the lytic agent increases in titer only in the presence and at the expense of young susceptible bacteria; moreover, older bacteria are known to adsorb lytic agent without undergoing lysis, thus decreasing the concentration of free lytic agent in solution.[19–21]

Protocol 6.

Effect of Relative Number of Old and Young Susceptible Bacteria on Number and Size of Plaques.

Molten 1 per cent agar, *cc.*	9	9	9	9	9	9	9
18 hr. culture of *B. dysenteriæ* Shiga, *cc.*	1.0	0.75	0.5	0.25	0.0	1	0.0
12 day culture of *B. dysenteriæ* Shiga, *cc.*	0.0	0.25	0.5	0.75	1.0	0.0	1
0.1 cc. of Laudman Shiga filtrate diluted.	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	0.0	0.0
Plates poured and incubated at 37°C. overnight.							
Maximum size of plaques, *mm.*	1	1	0.8	0.6	Discontinuous growth.	Even growth.	Discontinuous growth.
No. of plaques	240	105	82	20	Counting impossible.		

For these reasons it was thought that the relative concentration of young and old bacteria might also appreciably affect the appearance of plaques.

In order to investigate this question, emulsions of equal turbidity were prepared by suspending in physiological salt solution agar cultures of *Bacillus dysenteriæ* Shiga of 18 hours and of 12 days respectively. Varying amounts of each were added to the mixture of agar and lytic agents as indicated in Protocol 6.

[19] Adsorption experiment will appear in *The Journal of Experimental Medicine.*

[20] Jaumain, D., and Meuleman, M., *Compt. rend. Soc. biol.*, 1922, lxxxvii, 362.

[21] Prausnitz, C., and Firle, E., *Centr. Bakt., 1. Abt., Orig.*, 1924, xciii, suppl., 148.

This experiment shows that both the number and the size of plaques diminish as the proportion of old bacteria is increased. When old bacteria only were used, the growth was discontinuous and the plaques could not be distinguished.

Effect of Presence of Resistant Bacteria on the Appearance of Plaques.

Since, in the preceding experiment, it was found that the presence of old bacteria affects the appearance of plaques, it was thought useful to inquire into the effect of young resistant bacteria. For this pur-

Protocol 7.

Effect of Relative Number of Susceptible and Resistant Bacteria on the Number and Character of Plaques.

Molten agar 1 per cent, *cc.*	9	9	9	9	9	9	9
B. dysenteriæ Shiga susceptible, *cc.*	1.0	0.75	0.5	0.25	0.0	1.0	0.0
B. dysenteriæ Shiga resistant, *cc.*	0.0	0.25	0.5	0.75	1.0	0.0	1.0
0.1 cc. Laudman Shiga filtrate diluted	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	$1:10^6$	0.0	0.0
Plates poured and incubated at 37°C. overnight.							
Maximum size of plaques, *mm.*	1	1	1	1	—	Even layer of growth.	
Plaque count	70	100	70	73	No plaques.		
Character of plaques	Clear.	Fairly clear.	Faint.	Faint.			

pose suspensions of susceptible and of resistant bacteria were prepared from respective 18 hour cultures on agar, and an experiment identical in other details with that above was performed.

The presence of resistant bacteria had no effect on either the count or size of the plaques (Protocol 7). However, the plaques appeared to the naked eye less distinct, were faint, as the proportion of resistant bacteria was increased. The pale plaques were altogether indistinguishable under the low power of the microscope from the background of bacterial growth.

Effect of Specificity of Bacterial Substratum on the Size of Plaques.

In dealing with several lytic filtrates of different origin we have observed several instances in which a given filtrate exhibiting activity against two or more species of bacteria produced plaques of different size, depending on the bacterium used. In order to see whether it is the bacterial substratum or the lytic filtrate which is the decisive factor determining the size of plaques, the following experiment was performed.

Laudman Shiga filtrate, which was also active to a less extent on *Bacillus coli*, was increased in its potency for the latter organism by a

Protocol 8.

The Size of Plaques Produced by Certain Lytic Principles on Cultures of Heterologous Bacteria.

	Laudman Shiga filtrate.				*Coli* filtrate.			
	Produced from cultures of Shiga bacillus.		Produced from cultures of colon bacillus.		Produced from cultures of colon bacillus.		Produced from cultures of Shiga bacillus.	
Plated with	*B. dysenteriæ* Shiga.	*B. coli.*	*B. dysenteriæ* Shiga.	*B. coli.*	*B. dysenteriæ* Shiga.	*B. coli.*	*B. dysenteriæ* Shiga.	*B. coli.*
Maximum size of plaques, *mm*.....	1.2	0.6	1.2	0.6	0.6	0.6	0.6	0.6

series of passages in broth until its titer for colon bacillus reached 10^9 units per cc. Likewise a lytic agent, originally lytic for colon bacillus, was transferred in a series of passages with Shiga bacillus. A sufficient number of transfers was made in each case to eliminate by dilution all traces of the original lytic filtrate. The original Laudman Shiga lysin was then plated in 1 per cent agar with both *Bacillus dysenteriæ* Shiga and *Bacillus coli*. The filtrate resulting from "adaptation" of this lysin to *Bacillus coli* was also plated with both organisms. Similarly, the lytic agent originally active upon colon bacillus, and the product of its "adaptation" to Shiga bacillus, were each plated with both organisms as shown in Protocol 8. The concentration of agar was in all cases 1 per cent.

It appears from the above experiment that the Laudman lytic agent, whether produced in cultures of *Bacillus dysenteriæ* Shiga or in those of *Bacillus coli* retained the characteristic of forming larger plaques with *Bacillus dysenteriæ* Shiga and smaller plaques on plates of *Bacillus coli*. The lytic agent anti-*Bacillus coli* produced a plaque of about 0.6 mm. in diameter when grown with either *Bacillus dysenteriæ* Shiga or *Bacillus coli* irrespective of the culture from which the filtrate was obtained.

The size of the plaque formed on plates of a given bacterium is, then, a function of the lysin, and is retained without change through the process of so called adaptation.

DISCUSSION.

The results of our experiments indicate that the lytic principle acts as though it were present in the medium in the form of indivisible units. While it was suggested by d'Hérelle that these active units are actually organized living particles, the evidence so far produced by him in support of this view does not seem convincing. Since active filtrates consisting of water with a very small percentage of solids, comprising, in addition to active principle, salts, tissue extractives, metabolic products, and bacterial debris, can still be diluted 10^{10} times before reaching the limit of their activity, it is equally possible to suppose that the units of active agent are molecules of some inanimate material.

It is also possible that lytic substance is present in the medium in a state of true solution, and is merely adsorbed on bacterial debris and, being distributed with it, appears as being particulate itself. In such case the number of particles of bacterial debris present will determine indirectly the particulate concentration of active agent. This possibility is especially suggestive if it is remembered that the highest number of active units present in a lytic filtrate at the height of its activity is about 1×10^{10} per cc.—a concentration which very closely approaches the figure expressing the peak of the concentration of susceptible bacteria in a cc. of the medium.

Furthermore, the particles of lytic agent do not behave as if they were living organisms, as shown by the effect on them of comparatively slight changes in concentration of agar. Even as slight an increase

in consistency of the agar as that from 1 per cent to 2.5 per cent suppresses over 99 per cent of plaques. Moreover, it is difficult to reconcile the changes in the size of plaques, as brought about by various procedures in our experiments, with the view of d'Hérelle that the plaques represent the "colonies" of bacteriophage. For instance, if the size of the plaque depends on the progress of "invasion" of bacteria by the bacteriophage why should it be affected by the concentration of agar? Moreover, the more numerous the susceptible bacteria around the initial particle, the more they should be "invaded;" each of these, according to d'Hérelle, in turn should become a source from which the next generation of bacteriophage would invade the nearest bacteria, and in consequence the larger should be the plaque, or at least it should not be smaller. As a matter of fact, however, the greater the concentration of susceptible bacteria, the smaller are the plaques. Similarly it would be difficult to explain, on the basis of d'Hérelle's hypothesis, why both the number and the size of plaques decrease with the increase in proportion of *old* bacteria which are not subject to lysis, whereas the increase in concentration of young *resistant* bacteria leaves both the number and the size of plaques unaffected.

On the other hand, a logical explanation of these phenomena can be given on the basis of the observed tendency of agar and of old bacteria to adsorb the lytic agent, thus interfering with the progress of its diffusion. Since resistant bacteria do not adsorb the lytic principle, other conditions being equal, the presence of an excess of resistant bacteria does not interpose an obstacle for normal diffusion of the lytic agent—hence the difference in their respective effect on the size of the plaques.

SUMMARY AND CONCLUSIONS.

The experiments reported above confirm the fact that lytic principle is distributed in active solution in a state of indivisible units. This permits its quantitative evaluation by serial dilution, as well as by plating on agar. The latter method, however, often gives readings considerably lower than those obtained by the broth dilution method of titration. By varying the concentration of agar it has been possible to show that the discrepancy is due to adsorption of the lytic agent on agar. When the concentration of the latter is increased from 0.3 per

cent to 2.5 per cent the number of plaques of lysis is reduced more than 100 times. At the same time the average size of the plaques also decreases approximately to one-tenth of the original.

The size, as well as the number of plaques, has been found to depend also on the condition of the culture employed in titration. Thus, when the culture exposed to the action of lytic agent is composed of young susceptible bacteria, the greater the concentration of bacteria, the smaller the plaques.

When the culture is composed partly of young and partly of old susceptible bacteria, both the size and the number of the plaques are diminished with the increase in the relative concentration of old bacteria. On the other hand, presence in the culture of resistant bacteria does not affect either the size or the number of the plaques so long as the relative concentration of susceptible bacteria in the culture is sufficient to allow formation of them. The plaques appearing in the presence of a high concentration of resistant variants in the culture are relatively indistinct owing to overgrowth.

Under carefully controlled conditions the size of plaques is found to be determined by the character of the lytic filtrate. Thus in the case of lytic agents which act upon more than one bacterial species the size of the plaques remains constant, irrespective of the bacterial substratum used for the production of the active filtrate.

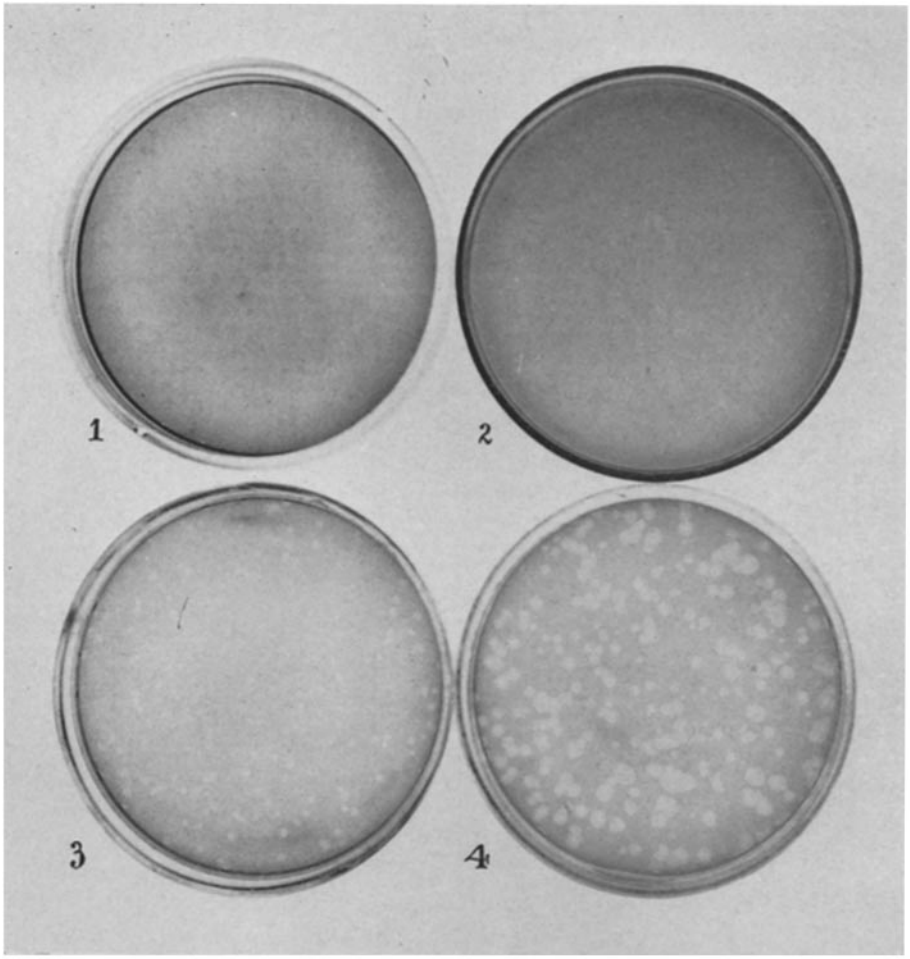
EXPLANATION OF PLATE 20.

FIG. 1. 4.5 per cent agar + 0.1 $\times$ 10^{-1} cc. or 100,000 $\times$ 10^{-7} cc. of phage—no plaques.

FIG. 2. 2.5 per cent agar + 0.1 $\times$ 10^{-4} cc. or 100 $\times$ 10^{-7} cc. of phage—130 plaques 0.25 mm. in diameter.

FIG. 3. 1 per cent agar + 0.1 $\times$ 10^{-7} cc. of phage—180 plaques 1.0 mm. in diameter.

FIG. 4. 0.5 per cent agar + 0.1 $\times$ 10^{-7} cc. of phage—281 plaques 2.5 mm. in diameter.



(Bronfenbrenner and Korb: Bacteriophage of d'Hérelle.)