INTERFERENCE BETWEEN BACTERIAL VIRUSES

III. The Mutual Exclusion Effect and the Depressor Effect[1,2]

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When a bacterium is simultaneously infected with two or more bacterial viruses (bacteriophages), only one of the viruses will grow, and the bacterium will be lysed after a time interval which is characteristic for the virus which grows. The other viruses, which are adsorbed by the bacterium but do not grow, are not recovered when the bacterium is lysed. Which one of the adsorbed viruses will grow in any one cell depends to some extent on accessory circumstances. In the case previously reported (Delbrück and Luria, 1942) virus gamma won out against virus alpha, except when infection with virus alpha occurred at least four minutes prior to infection with virus gamma.[3]

This phenomenon appears to be analogous to the interference phenomena of plant and animal viruses, the first example of which was described by McKinney (1929). The occurrence of the phenomenon with plant, animal, and bacterial hosts indicates that we are dealing with an aspect of the relation between viruses and their host cells of universal validity. It would seem that any real progress in our understanding of this phenomenon for one group of viruses should teach us something about all viruses.

In the present paper we wish to report further studies of interference between bacterial viruses. Our experiments confirm and extend without exception the previous finding that only one type of virus will be liberated from any one cell. This will be called the *mutual exclusion effect.*

In addition it is found that the virus which does not grow may nevertheless influence the course of events in such a way as to reduce the yield of virus of the type which grows. This will be called the *depressor effect.*

Most of the work to be reported in this paper was done with virus strains alpha and delta.[3] Interference between alpha and gamma has been reported previously. Some additional work on this pair, as well as on the pair gamma delta, will here be reported along with the alpha delta experiments.

METHODS

Most of the methods used in this work have been described previously. References will be given in the proper places. The only new method is the use of

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[2] We are greatly indebted to Dr. S. E. Luria for numerous friendly discussions throughout the period of this work and for actual collaboration in some of the earlier experiments.

[3] For a description of the viruses, see table 1 of Delbrück (1945a).

mixed indicator strains as a tester for the simultaneous liberation of different viruses from the same bacterium.

When studying the growth of a single strain of virus by the plaque count technique, any bacterial strain which is lysed by the virus will do. When one is working with a mixture of viruses and wishes to follow the titer of each virus, "indicator strains" are needed, i.e., strains which are sensitive to one virus and resistant to the other. Only one of the virus strains will give plaques when the mixture is plated with an indicator strain.

For some purposes it is desirable to have a method which will give a plaque only when *both* viruses are present in the same spot. This is achieved by plating the viruses with a mixture of the indicator strains. Each virus by itself will then form plaques which are overgrown by the indicator strain of the other virus. These plaques will be turbid. Only where both viruses are present will both indicator strains be lysed and a clear area be formed.

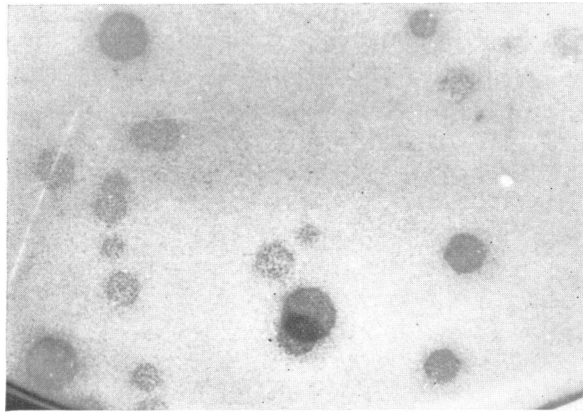

FIG. 1. A MIXTURE OF VIRUSES ALPHA AND DELTA PLATED WITH A MIXTURE OF THE INDICATOR STRAINS, A AND D

Figures 1 and 2 are photographs of plates on which a mixture of viruses alpha and delta was plated with a mixture of the two indicator strains. Strain A, the indicator strain for alpha, grows a little slower than strain D, the indicator strain for delta. Also, strain A gives a smooth growth while that of strain D is slightly granular. Figure 1 was taken when the indicator strains had grown about equally. The two types of plaques are distinguishable by the texture of the overgrowth. The alpha plaques are overgrown by D, and are granular, whereas the delta plaques are overgrown by A, and are smooth. One alpha plaque overlaps partially with a delta plaque. In the region of overlap both A and D are lysed, and this region is therefore completely clear. Figure 2 was taken one hour later from a similar plate. Here the alpha plaques are scarcely discernible because strain D has grown to almost the same turbidity as the background. There are several clear overlaps on this plate, one of which is almost perfectly concentric. This must have arisen from one alpha and one delta particle which happened to lie very closely together on the plate. This occurs rarely when a mixture of the free particles is plated.

Suppose, however, that a mixture of the two viruses is added in excess to a growing culture of bacteria of strain B, which is sensitive to both viruses. All bacteria will then adsorb particles of both kinds. If any such bacterium liberates at least one particle of virus of each kind, it will form a perfectly circular clear plaque when plated with mixed indicator strains. If, on the contrary, such a bacterium liberates only particles of one kind, the plaque which it forms on mixed indicator strains will be overgrown by the indicator strain for the other kind of virus, and will be turbid. The presence or absence of clear plaques is therefore a sensitive test of the validity of the principle of mutual exclusion.

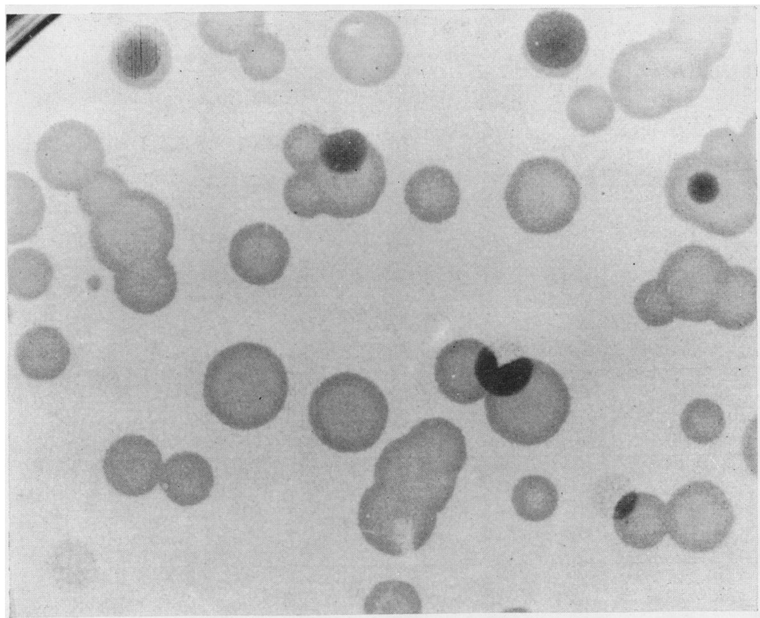

FIG. 2. A PLATE SIMILAR TO THAT SHOWN IN FIGURE 1, BUT TAKEN ONE HOUR LATER
For description see text

EXPERIMENTAL

Experiment 1. Multiple infection of growing bacteria of strain B with viruses alpha and delta, both added at the same time. The methods are described in Delbrück and Luria (1942).

In adsorption tube

6.6×10^7 bacteria per ml
68×10^7 alpha per ml
50×10^7 delta per ml

After five minutes the adsorption mixture is diluted 1:10,000 into broth. A sample is centrifuged and the supernatant is assayed for free virus.

Supernatant assay

27×10^7 free alpha per ml
14×10^7 free delta per ml

Therefore, adsorbed

41×10^7 alpha per ml = 60 per cent of input
36×10^7 delta per ml = 72 per cent of input

Multiplicity of infection

6.2 alpha per bacterium
5.5 delta per bacterium

Final titers

250×10^7 alpha per ml
380×10^7 delta per ml

Total yield (final titer minus free virus)

223×10^7 alpha per ml
366×10^7 delta per ml

Average yield per bacterium

34 alpha per bacterium
55 delta per bacterium

TABLE 1

Yields of alpha and delta for different multiplicities of infection

NUMBER OF EXPERIMENTS	AVERAGE MULTIPLICITY		AVERAGE YIELD PER BACTERIUM	
	alpha	delta	alpha	delta
5	5	4.5	22	60
3	3.6	6.3	6	76
1	6	3	51	38

The results show that both viruses grow but that the yield of each virus is about six times less than the corresponding yields when only one kind of virus is added. Plaque counts after 14 and 24 minutes have elapsed show that both kinds of virus are beginning to be liberated at 14 minutes and that liberation is complete at 24 minutes, as in the case of infection by either virus alone. Moreover, inspection of the adsorption tube shows noticeable clearing at 15 minutes and complete clearing at 17 minutes, as in the case of infection by either virus alone.

We conclude that all mixedly infected bacteria are lysed after the same latent period as for infection by either kind of virus alone. The average yield of virus of both kinds is depressed to about one sixth by the presence of the other virus.

Table 1 summarizes the results of nine similar experiments. Groups of experiments with closely similar multiplicities have been averaged. The first group corresponds to experiment 1, with nearly equal multiplicities of alpha and delta. In the other two groups the multiplicity is in favor of alpha or delta, respectively. It will be seen that the relative yields of the two viruses shift correspondingly, without, however, appreciably changing the total yield of virus.

Experiments with the pair gamma delta showed that both viruses grow in this combination also, but they grow much less here than in unmixed infections. The gamma yield is reduced to about one third, the delta yield to about one tenth of its value in unmixed infection. Delta, therefore, occupies an intermediate position between alpha and gamma with respect to interference.

Experiment 1 and the experiments listed in table 1 show clearly that there is interference between the growth of viruses alpha and delta when growing on our test strain of bacteria. It is also clear from these experiments that they cannot be explained by mutual exclusion alone, because in that case the yields of alpha and delta should add up to a total which is intermediate between the yields obtainable in experiments with either virus alone, i.e., between 200 and 300.

Experiment 1 does not even tell us whether the mutual exclusion principle holds for alpha delta interference. At first sight one might be led to believe that it does not hold, since in the mass culture both kinds of virus are liberated at the same time. It may be recalled that, in the experiments on alpha gamma interference, mutual exclusion was inferred from the fact that alpha and gamma liberation occurred at different times (13 and 21 minutes respectively), corresponding to the latent periods of these two viruses. This method of proof is not here available because alpha and delta happen to have the same latent period (13 minutes). Recourse must therefore be had to a method which tells us directly whether virus particles of both kinds may or may not be liberated from the same bacterium. Plating of mixedly infected bacteria with mixed indicator strains answers this purpose. In this method a clear plaque occurs only where a delta plaque and an alpha plaque start at the same point, i.e., either where a mixedly infected bacterium liberates at least one particle of each kind, or where an alpha plaque and a delta plaque accidentally overlap accurately. In order to reduce the chance for accidental overlaps, the number of free alpha and delta particles must be kept low in comparison with the number of mixedly infected bacteria. This can be achieved either by using low multiplicity (experiment 2) or by eliminating with antivirus serum the free virus particles after the adsorption period (experiment 3).

Experiment 2. Mixed infection with alpha and delta, both slightly in excess of the bacteria. Plating was done with mixed indicator strains after adsorption and before lysis.

In adsorption tube

9.2×10^7 bacteria per ml
16.5×10^7 alpha per ml
14×10^7 delta per ml

After 5 minutes a dilution of 1:5,000 is made into broth. A sample is centrifuged and the supernatant assayed for free virus.

Supernatant assay

6.8×10^7 free alpha per ml
3.1×10^7 free delta per ml

Therefore, adsorbed

10×10^7 alpha per ml
11×10^7 delta per ml

Multiplicity of infection

1.1 alpha per bacterium
1.2 delta per bacterium

From the multiplicity may be calculated, by Poisson's law, the fraction of the number of bacteria which were infected. With the data given above we find:

Bacteria infected with alpha *and* delta	47 per cent
with alpha only	20 per cent
with delta only	23 per cent
with neither	10 per cent

After 10 minutes five samples were plated with mixed indicator strains in such a dilution as to give 22 mixedly infected bacteria per plate. The plates showed numerous turbid plaques due to free alpha and delta particles, and to bacteria which liberated either alpha or delta. Clear areas appeared where plaques of different types overlapped. The mixedly infected bacteria should give clear plaques only *if they liberate particles of both kinds.* Twenty-two such clear plaques might be expected or 110 on all five plates together. Three plates showed no clear plaque at all; the other two showed one each. These may be ascribed to accidental overlaps.

Five similar experiments gave similar results. In all, 340 mixedly infected bacteria were plated in these experiments; observed were nine clear plaques.

One experiment with the pair gamma delta also gave no indication of bacteria liberating both gamma and delta.

We conclude that at most a few per cent, probably none, of the mixedly infected bacteria liberate particles of both kinds.

In these experiments the multiplicity of infection and the number of infected bacteria had to be kept low in order to reduce the chances for accidental overlaps of plaques caused by free virus particles.

In the following experiment the free virus particles were eliminated with antivirus serum. The method is described in the preceding paper (Delbrück, 1945b).

Experiment 3. Mixed infection with alpha and delta, both in multiple excess of the bacteria. Five minutes were allowed for adsorption. Free virus particles were eliminated after the adsorption period with a mixture of high titer sera against alpha and delta. Plating was done with mixed indicator strains after elimination of the free virus particles and before lysis.

In adsorption tube

4.3×10^7 bacteria per ml
60×10^7 alpha per ml
70×10^7 delta per ml

Adsorption for 5 minutes. Multiplicity of infection

6 alpha per bacterium
10 delta per bacterium

After 5 minutes a dilution of 1:50 was made with broth containing antialpha serum (diluted 1:20) and antidelta serum (diluted 1:100) and incubated for 4 minutes.

After 9 minutes there was a further dilution of 1:1,000 with broth. From this dilution samples of 0.055 ml were plated by the agar layer technique on each of five plates, with mixed indicator strains. Each plate thus received 47 mixedly infected bacteria. The plaque counts from the plates are listed in table 2. They show, first of all, that there was only one clear plaque out of a total of 263. The clear plaque may have been an accidental overlap. There are, therefore, no bacteria which liberate particles of both kinds. Second, the alpha and delta

TABLE 2

Plaque counts from experiment 3

PLATE NO.	TURBID ALPHA PLAQUES	TURBID DELTA PLAQUES	CLEAR PLAQUES
1	21	25	0
2	20	38	0
3	27	36	0
4	20	34	1
5	15	28	0
Average.............	20.5	32	0.2

Sum of alpha and delta plaque counts, 52.5.
Number of bacteria per plate, 47.

plaque counts add up to a total in close agreement with the total number of mixedly infected bacteria on each plate. Therefore, *every* bacterium liberates *either* alpha *or* delta.

Experiments 2 and 3 show that a bacterium infected with alpha and delta will liberate either alpha or delta particles (mutual exclusion effect). Since experiment 1 showed that the total yield is reduced, it follows that the *average* yield of virus from such a bacterium is smaller than it would have been from a bacterium infected by only one kind of virus (depressor effect). In the case of infection by one kind of virus we know that the burst sizes of individual bacteria vary widely (cf. figure 1 of Delbrück, 1945a). For an understanding of the depressor effect it is then of first importance to find the distribution of burst sizes in the case of interference. The reduction of the average burst size might be due either to a proportional reduction of all burst sizes, or to an extreme reduction of the bursts from some of the bacteria while others have normal yields.

Individual burst sizes may be studied by a combination of the techniques used in the preceding experiments and those described by Delbrück (1945a).

Experiment 4. Mixed multiple infection with alpha and delta; dilution after 4 minutes' adsorption. Sixty samples, containing on the average about 0.5 bacteria each, are distributed before the beginning of lysis, incubated until lysis is complete, and are then plated with mixed indicator strains.

In adsorption tube

8×10^7 bacteria per ml
70×10^7 alpha per ml
45×10^7 delta per ml

After 4 minutes the adsorption mixture is diluted 8×10^6-fold in broth. Sixty samples of 0.048 ml each are distributed into small tubes and incubated 30 minutes. From a suitable dilution of the adsorption mixture the percentage of adsorption and the multiplicities are determined.

Adsorbed

44×10^7 alpha = 63 per cent of input = 5.5 per bacterium
39×10^7 delta = 87 per cent of input = 4.9 per bacterium

Contents of each sample

0.48 bacteria, mixedly infected
1.2 free alpha
0.29 free delta

After 30 minutes all samples are plated with mixed indicator strains and the plates incubated for six hours. At this time both types of plaques (both turbid) show up distinctly, and the difference between alpha and delta plaques is easy to tell by the difference in texture of the overgrowth.

The plaque counts are listed in table 3 and will be analyzed in some detail.

For each kind of virus there are plates showing plaques obviously due to bursts, and other plates which show none, and a third group which shows very few plaques of this kind. The plaques on this last group of plates must be due to free particles, and not to bacteria yielding only a few virus particles, for the following reasons:

(a) There are 13 plates with alpha bursts and 19 plates with delta bursts. Correcting for accidental doubles we obtain an average of 0.62 bursts per sample. This is in fair agreement with the input of 0.48 infected bacteria per sample. There are, therefore, no bacteria left that might account for the plates with few plaques.

(b) Taking the plates without alpha bursts, we find the distribution given in table 4. This leads to an average of 1.34 plaques per plate, in close agreement with 1.2, the number of free alpha particles per sample calculated from the plaque count after adsorption. The third column in table 4 gives the expected number of plates with a given number of plaques, calculated as Poisson distribution with 1.34 as the average value. It will be seen that the calculated and the experimentally found distribution agree closely. There is, therefore, sufficient reason for ascribing the plates with few plaques to free virus particles.

The analysis of the small delta counts is also consistent with this interpretation. Table 4 lists the distribution, which leads to an average of 0.24 plaques per plate, in close agreement with 0.29 free delta particles per sample. Here, too, the calculated Poisson distribution agrees satisfactorily with the one actually found.

Turning now to the bursts we note first of all that there is no correlation between the occurrence of alpha and delta bursts. The number of plates which

TABLE 3

Alpha and delta plaque counts from sixty samples of experiment 4

SAMPLE NO.	PLAQUES		SAMPLE NO.	PLAQUES	
	alpha	delta		alpha	delta
1	2	126	31	1	108
2	117	0	32	0	0
3	87	0	33	70	102
4	0	73	34	1	24
5	2	1	35	1	0
6	1	0	36	2	0
7	3	126	37	3	0
8	39	0	38	0	201
9	1	30	39	1	0
10	43	13	40	1	160
11	0	0	41	0	0
12	1	0	42	1	0
13	2	44	43	2	0
14	1	24	44	152	0
15	2	0	45	4	76
16	1	2	46	1	0
17	84	1	47	2	0
18	0	1	48	8	1
19	2	1	49	32	124
20	22	102	50	103	0
21	2	0	51	2	0
22	3	0	52	1	110
23	2	1	53	2	74
24	0	0	54	97	0
25	1	0	55	3	0
26	0	12	56	0	0
27	0	81	57	1	0
28	3	0	58	0	0
29	2	0	59	3	2
30	0	0	60	42	0

show both alpha and delta bursts should be 3.8 for random coincidence. Actually four such plates are found, nos. 10, 20, 33, and 49. This is perhaps the most striking illustration of the mutual exclusion effect. We can go one step further and assert that not a single alpha particle is liberated in association with a delta burst. For, if an alpha particle were liberated in association with a delta burst, then the average count of "free" alpha particles in samples *with* delta bursts

should be slightly higher than the number of free alpha particles in samples *without* delta bursts. Analysis of table 3 gives

1.27 alpha particles per plate, on plates with delta bursts
1.37 alpha particles per plate, on plates without delta bursts

These findings reinforce the proof of the mutual exclusion effect.

TABLE 4

Distribution of plaques due to free particles (experiment 4)

NUMBER OF PLAQUES	ALPHA		DELTA	
	Number of plates			
	found	calculated	found	calculated
0	12	12.3	33	32.2
1	15	16.5	6	7.7
2	13	11.1	2	1.0
3	6	5.0	0	0.1 (for 3, 4, >4)
4	1	1.7	0	
>4	0	0.4	0	
Total..........	47 plates, 63 plaques		41 plates, 10 plaques	
Average........	1.34 plaques/plate		0.24 plaques/plate	

TABLE 5

Alpha and delta burst sizes (experiment 4)

ALPHA BURSTS	DELTA BURSTS
8	12
22	13
32	24
39	24
42	30
43	44
70	73
84	74
87	76
97	81
103	102
117	102
152	108
	110
	124
	126
	126
	160
	201

Finally, we come to the burst sizes. They are listed in order of size in table 5. Two things are at once obvious, viz., first, that the average burst size is much smaller than that for unmixed infection, and, second, that the distribution of

burst sizes is as irregular as that of unmixed infection. Both large and small burst sizes seem to be cut back by about the same factor. Taking into account accidental doubles we find for the average burst size—

For bacteria liberating alpha	62 alpha particles per burst
For bacteria liberating delta	71 delta particles per burst

Summing up the results of experiment 4 we obtain the following: (a) all bacteria are lysed; (b) each bacterium liberates either alpha or delta, none both; (c) about one third of the bacteria liberate alpha particles, and two thirds of the bacteria liberate delta particles; (d) the bacteria liberating alpha particles do so in amounts ranging between 8 and 152, with an average of 62 per bacterium; (e) the bacteria liberating delta particles do so in amounts ranging between 12 and 201, with an average of 71 per bacterium.

Very similar results were obtained in four similar experiments. This material is not large enough to yield a reliable burst size distribution curve, but the results show clearly that all bacteria are similarly affected. There is a general reduction of all burst sizes.

In quantitative terms the problem may now be stated as follows: A bacterium which has adsorbed n_{alpha} alpha particles and n_{delta} delta particles has a probability p_{alpha} of liberating alpha particles and p_{delta} of liberating delta particles. The fact that each bacterium must liberate either one or the other is expressed by the equation

$$p_{alpha} + p_{delta} = 1$$

For $n_{alpha} = n_{delta} = 5$ we find $p_{alpha} = \frac{1}{3}$ and $p_{delta} = \frac{2}{3}$. For other multiplicities, the split will be different.

A bacterium which liberates alpha particles will liberate on the average N_{alpha} alpha particles, and a bacterium which liberates delta particles will liberate on the average N_{delta} delta particles. In experiment 4, we found $N_{alpha} = 62$ and $N_{delta} = 71$. However, these quantities, too, will depend on the multiplicities. The further analysis, then, should center around the determination of the four functions, p_{alpha}, p_{delta} (the split of the bacteria into alpha yielders and delta yielders), and N_{alpha}, N_{delta} (the average yield of each kind of bacteria), in their dependence on the multiplicities, and, as we shall see, on timing relationships between infection by alpha and by delta.

At first sight it would seem that we had the techniques for this purpose in hand. p_{alpha}, p_{delta} might be determined, as in experiment 3, by the use of antisera and plating before lysis, and N_{alpha}, N_{delta} by plating after lysis.

Many experiments of this kind were performed. They revealed, however, two unexpected phenomena, which throw new light on the interference mechanism, but which at the same time frustrate to some extent the program described in the last paragraphs.

The new phenomena are due to direct effects of antibacterial antibodies (agglutinins) on the one hand, and of antivirus antibodies on the other hand, on interference. Of the two the action of agglutinins is simpler.

ACTION OF AGGLUTININS ON INTERFERENCE

In the preceding paper (Delbrück, 1945b) the action of agglutinins on the growth of a single virus was analyzed and it was found that at high serum concentration the action consists of preventing the adsorption of virus and, at low serum concentration, of delaying the action of adsorbed virus. These effects show up only if the serum is added prior to the addition of virus. In the study of mixed infection, they show up most strongly if the antiserum is added *after* the adsorption of one virus and *before* the adsorption of the other.

Experiment 5. Single infection with alpha, multiple with delta. Virus delta is added three minutes after virus alpha. In between, namely, two minutes after alpha and one minute before delta, antibacterial serum in various dilutions is added. In each case the step size of alpha growth is measured.

The results are listed in table 6. If no serum is added, alpha growth is almost completely suppressed by delta. The step size is four. Intermediate addition of antibacterial serum in high concentration has the effect of blocking off delta,

TABLE 6

Action of antibacterial serum on the interference between virus alpha and virus delta
Serum added two minutes after alpha and one minute before delta

SERUM DILUTION	STEP SIZE OF ALPHA GROWTH
no serum	4
50	40
100	40
200	40
400	25
1000	5
2000	5

thereby permitting full growth of alpha. The step size is then forty. This holds for serum dilutions up to 1:200. At 1:400 alpha growth is still very appreciable; at 1:1,000 it is as suppressed as in the absence of serum. Titration of the serum by this method, therefore, gives an end point around 400. This is higher than the titer 100 which may be read from table 1 of the preceding paper (Delbrück, 1945b), the "antiadsorption titer," but agrees with the titer 320 to 640 of table 2, the "lysis-delaying titer."

It follows from these results that interference, in the arrangement of experiment 5, is a sensitive indicator of antibacterial antibodies. As mentioned previously, it was in experiments of this type that the effect was first noted. The only difference was that in the early experiments antivirus serum was employed which had a weak antibacterial activity because of the impurity of the antigen used for immunization of the animals. Table 7 lists some of the experiments which served to discover the nature of the effect. The experiments were run in pairs, the members of one pair differing only in the material added intermediately between the two viruses.

In experiments 6 and 7 no serum is compared with normal rabbit serum. In the absence of serum, suppression of alpha growth is almost complete. Normal serum relieves the suppression only to a very slight extent. In experiment 8 no serum is compared with antialpha serum. The antialpha serum greatly *increases* the growth of alpha. Experiment 9 shows that this paradoxical result was not due to the antialpha component of the serum but to its antibacterial component, since the effect disappears after absorption of the serum with bacteria. Experiment 10 gives a direct comparison of unabsorbed and absorbed serum, which confirms this conclusion.

With regard to interference, these experiments show that agglutinins can check the interfering action of the second virus, delta, even when they do not prevent its adsorption.

TABLE 7

Action of various antivirus sera and of normal serum on the interference of virus delta with the growth of virus alpha

EXPERIMENT NO.	TYPE OF SERUM (ALL EXPERIMENTS DONE WITH SERUM DILUTION 20)	STEP SIZE OF ALPHA GROWTH
6	none	2
	normal rabbit serum	4
7	none	2
	normal rabbit serum	6
8	none	2.5
	strong antialpha	20
9	none	3
	antialpha absorbed with bacteria	6
10	antialpha serum	30
	absorbed antialpha serum	6

With regard to the use of antivirus sera for the elimination of free virus after adsorption these experiments show that the antibacterial component must be removed from the serum if it is to be added before adding one of the viruses.

ACTION OF ANTIVIRUS SERUM ON INTERFERENCE

There is, however, also an effect of antivirus serum on interference which is caused by the antivirus antibodies, and which is manifest even when the antiserum is added several minutes after the virus. The essential features of the effect are well exhibited by the next experiment.

Experiment 11. Multiple infection with alpha and delta, added simultaneously. Three and one-half minutes were allowed for adsorption, then 4 minutes for exposure to a mixture of strong antialpha and antidelta serum. Both sera had previously been absorbed with bacteria. Plating with indicator strains

occurred both before and after lysis. A control without addition of antisera was run in parallel.

Plaque counts of the serum-treated culture *before* lysis showed the split of the bacteria into alpha yielders and delta yielders in the usual ratio. Plaque counts *after* lysis showed yields of alpha and of delta which were *greater* in the serum-treated culture than in the control (table 8).

The effect on alpha growth is more pronounced than that on delta growth. This was confirmed by other similar experiments, which indicated that the increase in alpha yield is due to the antidelta serum. The effect may be described in this way: Delta exerts a depressor action on the alpha yield. This depressor action is partly inhibited by antidelta serum added *after adsorption of delta.*

In the preceding paper (Delbrück, 1945b), we described experiments on the action of antivirus serum on the growth of a single virus. No effect of antiserum added *after adsorption* was found. Here we do find an effect of antivirus serum after adsorption. In a general way this result shows that interference is a more sensitive indicator for the details of virus growth than is the technique of the one step growth of a single virus.

TABLE 8

Effect on interference of antivirus serum added after adsorption of the viruses

	BACTERIA EXPOSED TO MIXED ANTIVIRUS SERA AFTER ADSORPTION	CONTROL
N_{alpha}	50	15
N_{delta}	120	75

Specifically our result shows that antivirus sera cannot be used to study the quantitative details of the mutual exclusion effect and the depressor effect, since the sera not only eliminate free virus but also modify the course of the phenomenon to be studied.

TIMING

It is possible, however, to progress a little further with the techniques now available, without the use of antisera. If the time interval between the addition of two viruses to a bacterial culture is increased, the probability that the second virus will establish itself in the bacterium decreases rapidly. For alpha delta interference the second virus does not grow at all (whether it be alpha or delta) if added more than four minutes after the first. In alpha gamma interference (Delbrück and Luria, 1942) there is a little gamma growth even when gamma is added six minutes later than alpha. There is a definite time interval for each pair of viruses which suffices for the establishment of the first virus in all infected bacteria. The second virus can, however, still exert a depressor action on the first one. This may be shown by experiments of the following kind:

Experiment 12. Multiple infection with the first virus at time zero. After 4 minutes a dilution of 1:2,000 is made with broth. Every few minutes a sample

from this tube is put into one of a series of tubes containing the second virus, the depressor, in high concentration. After lysis these tubes are assayed for the first virus.

If transfer into the tube containing the depressor is made after completion of lysis, the depressor has no effect. The yield is then as high as in the absence of

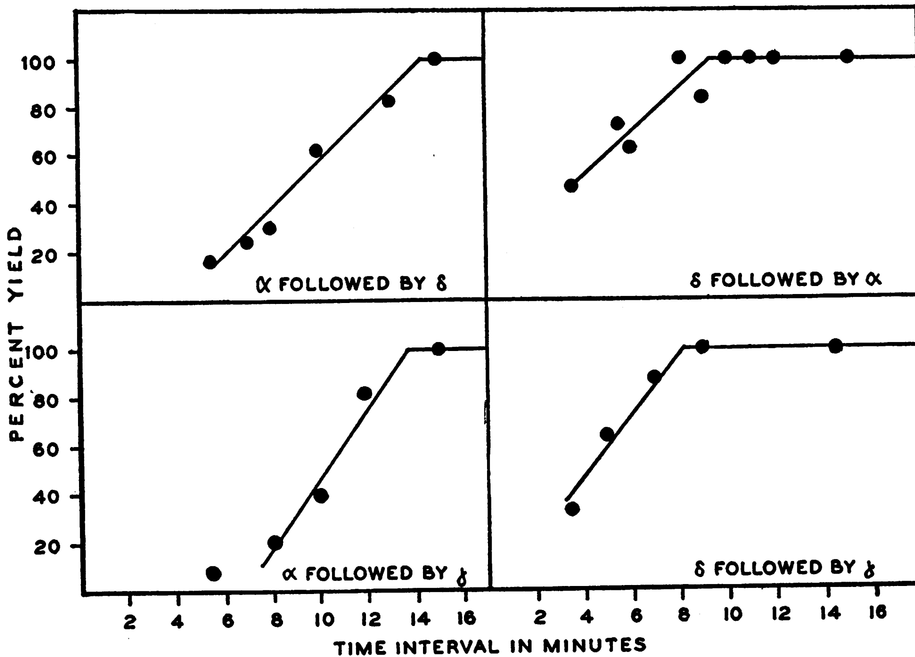

FIG. 3. DEPRESSOR EFFECT

The yield of the virus added first is plotted as a function of the time interval between the addition of the two viruses. The yields are given as percentages of the controls in which no second viruses were added.

TABLE 9

End point of depressor action

FIRST VIRUS	DEPRESSOR	END POINT
		min
alpha	delta	14
alpha	gamma	14
delta	alpha	9
delta	gamma	8

the depressor. The plaque counts from the earlier transfers show the percentage of depression in its dependence on the time at which the depressor was added.

Figure 3 shows the results of experiments on four combinations of viruses, viz., alpha followed by delta or gamma, and delta followed by alpha or gamma. In all four of these cases the depressor effect diminishes only gradually when the

time interval between the addition of the two viruses is increased. Of particular interest is the "end point," that is, the time interval beyond which there is no depressor effect. These end points, as read from figure 3, are listed in table 9. It will be seen that the end point is independent of the nature of the second virus. Thus, for alpha the end point is at 14 minutes, slightly after the beginning of lysis, with either delta or gamma as depressor. Similarly, for delta the end point is at 8 to 9 minutes, for either alpha or gamma as depressor. It would appear that the end point characterizes a stage in the growth of the first virus.

DISCUSSION

Our experiments show some details of the interference phenomenon which were not apparent from the previous work. In the first place, they extend qualitatively and quantitatively the validity of the mutual exclusion principle. Qualitatively we find the principle verified for the pairs alpha delta and gamma delta. Quantitatively we find for the pair alpha delta that the exclusion mechanism operates with astonishing efficiency. Certainly not more than 1 per cent of the mixedly infected bacteria liberate particles of both kinds. These experiments were performed with growing cultures of bacteria. Such cultures contain a fair proportion of bacteria which are about to divide. Our experiments show that such pairs must react to mixed infection as one unit right up to the moment of physical separation. This finding may be an important clue to the mechanism of exclusion. It speaks strongly against the hypothesis of the key enzyme (Delbrück and Luria, 1942). This hypothesis assumed that the cell contains an enzyme which is completely engaged by a single virus particle. When the cell divides, this enzyme must be present in both daughter cells. Therefore, this hypothetical enzyme would have to be doubled some time before the division of the cell. If the cell were mixedly invaded after the division of the enzyme, the hypothesis would lead to the prediction that both viruses would grow, and that, when the cell was plated before lysis, it would give rise to a clear plaque in experiment 3. The failure to find such clear plaques in appreciable number, therefore, speaks against the hypothesis of the key enzyme.

Another mechanism of mutual exclusion was suggested by electron microscope studies (Luria, Delbrück, and Anderson, 1943). The micrographs seemed to show that, in multiple infection, most of the adsorbed virus particles do not penetrate the cell. One might assume, therefore, that the entrance of the first virus makes the cell wall impermeable to other virus particles, just as the fertilization of an egg by one spermatozoon makes the egg membrane impermeable to other spermatozoa. This may be termed the "penetration hypothesis."

In order to explain our results as to the efficiency of the exclusion mechanism on the basis of the penetration hypothesis, one has to assume that the cell membrane reacts as a unit right up to the moment of mechanical separation of the daughter cells; that the change in permeability spreads very rapidly over the entire cell surface as soon as one virus particle has entered the cell; and further, that several minutes may elapse between adsorption and penetration, since, in alpha gamma interference, virus gamma can be added four minutes after virus alpha and yet exclude virus alpha. Differences of viruses with respect to their

exclusion power would be interpreted as differences in their rates of penetration. This is an attractive feature of the hypothesis since it means that the exclusion power has nothing to do with the growth rate of the virus after it has entered the cell. In alpha gamma interference, for instance, the slower-growing gamma has the greater exclusion power. This becomes understandable on the hypothesis that the exclusion power is determined by the rate of a particular phase of the total growth process, namely, the rate of penetration. The rate of this phase may be great even though the over-all rate is small.

The hypothesis of penetration implies that a group of viruses, all members of which attack the same host, may be arranged in linear order with respect to the exclusion power, since the exclusion power should be an image of the penetration rate. For instance, if we have three viruses, A, B, C, and we find that $A > B$ and $B > C$ with respect to exclusion power, we should find $A > C$. Our group, alpha gamma delta, fulfills this condition, with gamma > delta > alpha. Virus delta is weaker with respect to exclusion than gamma, and stronger than alpha, and the exclusion of alpha by gamma is stronger than any other combination. Any new virus that may be added to this group should be placeable without ambiguity in the series when tested in combination with all previous members of the group. Such tests are now under way.

Qualitatively the penetration hypothesis accounts nicely for the unspecific nature of the exclusion mechanism. Exclusion occurs between any pair of viruses which has been tested, whether the viruses are related or not. Further, virus which has been inactivated by ultraviolet light retains its exclusion power (Luria and Delbrück, 1942). There is only one possible exception to the rule that only one type of virus is liberated by one cell, which is suggested by Luria's experiments on spontaneous mutations of bacterial viruses (Luria, 1945). Luria finds that the mutations occur intracellularly, during the growth of the virus, and his experiments indicate that a bacterium in which such a mutation has occurred may liberate particles of both the unmutated and the mutated kind. This is just what one would expect on the penetration hypothesis, since the second virus, if it is created within the cell by mutation, cannot be excluded by a change in permeability of the cell membrane.

We turn now to the depressor effect. We have seen that the virus which is excluded from growth is not necessarily without influence on the course of events. In the cases of alpha and of delta the yields of virus are reduced by the presence of the excluded virus. This effect appears to be specific in the negative sense, i.e., it occurs only if the excluded virus is dissimilar to the growing one. There is no depressor effect when the growing virus and the excluded one are identical, i.e., in self-interference. There also appears to be no effect on virus gamma by any of the other viruses tested. The depressor effect diminishes gradually when the time interval between the addition of the two viruses is lengthened. There is an upper limit for this time interval beyond which there is no depressor effect, and this upper limit appears to be fixed for each virus. It is less than 4 minutes for gamma, 8 to 9 minutes for delta, and 14 minutes for alpha.

One is tempted to interpret the depressor effect as a competition of the viruses

for a common substrate. Such an interpretation would account for the absence of the depressor effect in self-interference. The time limit for the effect would indicate the time at which all the available substrate has been used up by the first virus.

In order to reconcile this interpretation of the depressor effect with the penetration hypothesis one must assume that the excluded virus can compete for substrate even though it does not enter the cell; further, that the substrate in question is at or near the cell membrane and is available to both the virus particle which penetrates and to the excluded particles.

Antivirus serum, added several minutes after adsorption of both viruses, diminishes the depressor effect. This result speaks strongly in favor of the idea that the depressor effect is caused by virus particles located on the surface of the bacterium. In the preceding paper (Delbrück, 1945b), we found that antivirus serum fails to influence the growth of a single virus. This indicates that the antibody cannot follow the virus particle into the host cell. The action of antivirus serum on the depressor effect, therefore, should be due to the action of antibodies on virus particles which are located on the surface of the bacterium.

Summing up our analysis we arrive at the following working hypothesis. Mutual exclusion is caused by impermeability of the cell membrane induced by the first virus particle which penetrates the membrane. Each virus strain has a characteristic penetration time. The change in permeability occurs suddenly at the end of this time and is established rapidly for the entire cell membrane. A dividing cell reacts as a unit up to the moment of separation of the daughter cells. The virus which is barred from entry into the cell can nevertheless compete with the intracellular virus for a common substrate. It converts the substrate in an irreversible reaction into a product which is characteristic for each virus. This competition for substrate is the cause of the depressor effect which occurs only between dissimilar viruses. There is an upper time limit, characteristic for each virus strain, beyond which there is no depressor effect. This time limit marks the end of the period during which the first virus utilizes the available substrate. Antivirus serum can inhibit the action of the adsorbed virus on the substrate. These hypothetical relationships are represented schematically in figure 4.

It would seem that the penetration hypothesis, suitably elaborated, can account for our results. We do not, however, consider that our results in any way prove the validity of the penetration hypothesis.

Recently a number of studies of interference between animal viruses have appeared. Henle and Henle (1943) found suppression of the growth of influenza virus A in chick embryos which had received a previous injection of inactivated virus of the same strain. Ziegler and Horsfall (1944) and Ziegler, Lavin, and Horsfall (1944) confirmed these findings and extended them to cross-interference tests between active and inactive viruses of strains A and B and of swine influenza. Schlesinger, Olitsky, and Morgan (1944) in a series of striking experiments could demonstrate interference, in the brain tissue of guinea pigs, between the serologically unrelated viruses of Western and Eastern equine encephalomyelitis and of vesicular stomatitis.

These studies, as well as older studies on plant virus interferences, point strongly to a fundamental similarity of the mechanisms of interference of viruses in animal tissue, plant tissue, and in bacteria. By implication they strengthen the idea of the homology of these three groups of viruses. It should not be overlooked, however, that the analysis of the interference phenomenon in animal and plant tissue has not yet been carried beyond the crude outlines. In none of the cases of animal or plant virus interference do the experimental results permit us to infer the validity of the mutual exclusion principle. They could all be explained by slight modifications of the depressor effect. To decide this issue it would be necessary to analyze the yields from individual cells or cell components. The only attempt in this direction is that of Anderson (1942), who could demonstrate cytologically the coexistence of typical inclusion bodies of two different viruses in the same cell. It may be very significant, however, that she found dually infected cells only for combinations of viruses in which one strain causes intranuclear, the other intracytoplasmic, inclusions.

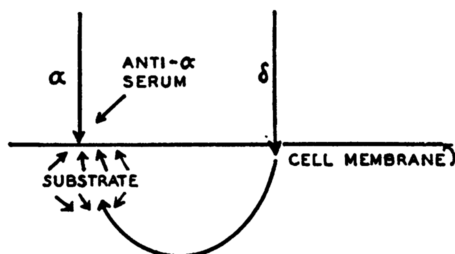

FIG. 4. SCHEMATIC REPRESENTATION OF THE PENETRATION HYPOTHESIS
Virus delta has penetrated the cell membrane, which thereupon has become impermeable to virus alpha. Virus delta and virus alpha compete for a common substrate. The action of virus alpha on this substrate can be inhibited by antialpha serum.

Ziegler, Lavin, and Horsfall (1944) advance the hypothesis that the interference between influenza viruses, observed by them, is due to quantitative saturation of the receptor substance discovered by Hirst (1942). A similar hypothesis would certainly not be applicable to our results, since there is no interference between bacterial viruses with respect to adsorption. The hypothesis advanced by Ziegler, Lavin, and Horsfall, therefore, is incompatible with the assumption of the essential similarity of the interference phenomena observed with animal, plant, and bacterial viruses.

SUMMARY

Bacteria which are simultaneously infected with viruses alpha and delta are lysed after a time interval of 13 to 17 minutes. This is the same time interval as that for unmixed infection by either virus. Every mixedly infected bacterium liberates either virus alpha or virus delta, none both (fewer than 1 per cent of the bacteria). This is called the "mutual exclusion effect." For equal multiplicity of infection with both viruses, about one third of the bacteria liberate virus alpha, two thirds liberate virus delta. A group of viruses which attack one host can be arranged in a series according to their exclusion power.

In mixed infections with alpha and delta the average yield of virus from a bacterium is very much less than in unmixed infection. The excluded virus

depresses the yield of the successful virus. The depressor action diminishes gradually when the time between the addition of the two viruses is increased. There is a critical time interval beyond which there is no depressor action. The length of this time interval depends on the nature of the first virus and is independent of the nature of the virus which acts as depressor.

Antibacterial serum added in high concentration before the second virus prevents adsorption of the second virus. If added in low concentration the antibacterial serum does not prevent adsorption of the second virus but diminishes its interfering action.

Antivirus serum, added after adsorption of a virus, diminishes its depressor action.

A working hypothesis, the "penetration hypothesis," is elaborated. The hypothesis assumes: (a) that the penetration of the first virus into the cell makes the cell membrane impermeable to any other virus; (b) that each virus has a characteristic time of penetration and that the change in permeability occurs at the end of this time interval uniformly for the entire cell membrane; and (c) that dividing cells act as a unit up to the moment of separation.

The depressor effect is interpreted as competition for a common substrate between the virus which penetrated into the cell and the excluded virus.

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