

MUTANTS OF A BACTERIOPHAGE OF BACILLUS MEGATHERIUM

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PLATES 27 AND 28

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Bacillus megatherium, strain 899a (lysogenic) of den Dooren de Jong (1) grows in association with a phage (T) which will produce turbid plaques on a sensitive strain of *B. megatherium*. Gratia (2) has shown that another phage (C), which produces clear plaques, can also be isolated from these cultures. We have identified four other plaque-type phage mutants. All but one of these newly isolated strains may be classified as turbid (T) or clear (C) and differ from the parental strain by their minor characteristics; *i.e.*, large and bright (br), minute (m), or very minute (vm). One mutant (S) has not as yet been satisfactorily classified. This report deals with both the method used for the isolation of these strains, and with the results of experiments in which bacteria were infected with pairs of these viruses.

Plaque mutants of phages have been described previously (3-5) and it has been shown (6, 7) that four phages are produced after simultaneous inoculation into a sensitive *Escherichia coli* culture of large amounts of two mutant phages in high phage-bacterium ratios. These include the two original mutants, the wild type, and a phage exhibiting characteristics of both original types.

This kind of reaction has not been observed with the *B. megatherium* phages. In our experiments the only recombinations observed were produced when a C of one minor type was mixed with a T of a second minor type. The new phages found in all cases were a C of the second minor type and a T of the first minor type.

Materials and Methods

The bacterial cultures used in these experiments, 899a (lysogenic) and KM (sensitive), have been adequately reported previously (8, 9), including methods used in their maintenance, preparation, and enumeration. Phage strains were prepared by multiple single plaque transfers on 5 per cent peptone agar to make certain of purity. Finally, lysates were prepared, filtered through one-half an EK Seitz pad, and stored at 4°C. These filtered lysates are bacteriologically sterile and stable over several months' time, except in one case to be noted below.

The mixed infection experiments were done in the following manner. To 10 cc. of 5 per

cent peptone (pH 7.2), containing 5×10^8 infective units of each of the two phages per cc., is added 5×10^7 sensitive *B. megatherium* cells per cc. This tube is shaken (200 cycles per minute) in an incubated water bath at 34°C. After 20 minutes, the mixture is chilled and centrifuged sufficiently to pack the bacteria at the bottom of the tube. The supernate is removed and titered for phage while the bacterial sediment is transferred to 10 cc. of 5 per cent peptone in a fresh tube (tube A). An aliquot of tube A is diluted in 5 per cent peptone to a total of 10^{-4} in a 10 cc. volume (tube B). Both tubes are placed in the water bath shaker, and while tube A is followed by colorimetry, the diluted tube (B) is titered for phage at time 0, 60, 120, and 150 to 180 minutes.

	mg./ml.
<i>Asparagine media</i> (9):	
Asparagine.....	10
Ferrous ammonium sulfate.....	0.02
MgSO ₄	0.04
CaCl ₂	0.01
Dissolve in 0.01 M pH 7.4 PO ₄ buffer, adjust to pH 7.4, boil, filter and autoclave.	

RESULTS

Origin of Strains.—All but one of the strains reported here were isolated in the course of a study of the changes taking place in cultures of *B. megatherium* (KM) infected with C phage. These cultures had regenerated after lysis and were subcultured (1/10 dilution) to fresh 5 per cent peptone or asparagine medium daily. Usually, by the 3rd day of the experiment, C(m) virus appeared in the culture in high titer. This has been isolated several times. By the 5th day the entire culture seemed to have stabilized, its growth approximated that of the control, and titers of C and C(m) virus remained fairly constant, around 10^7 phage per cc. Attempts were made to isolate lysogenic cells carrying C or C(m) virus with no success, but three strains of lysogenic bacteria were isolated which produced variant viruses (T(m); T(vm); S). On one occasion only, another mutant was observed on a plate containing 200 T plaques in which one plaque was brighter than the others. This was isolated and proved to be a new strain and is designated T(br).

By crossing any mutant T with the original C, the clear analogue of that mutant strain could be found. Both T and C analogues are described herein. The terminology retains the original C and T designations of Gratia for the major characteristics. These are followed by abbreviations of descriptive adjectives for the minor characteristics as explained below. (Also see Figs. 1 through 5.)

Description and Classification of Plaques

- T (turbid), the original virus, is produced by cultures of *B. megatherium* 899a and is recognized as a large turbid plaque with a minute crater-like hole in the center surrounded by a small ring of higher turbidity. (Figs. 1, 2, 5)
- C (clear), first isolated by Gratia (2), appears with regularity in cultures of T virus. Its plaque is somewhat larger than T and consists of a clear disc with a thin granular halo. (Figs. 1, 4, 5)

- T (br) is a bright version of T having the same basic structure. It differs in being slightly larger and having definitely less turbidity. The difference is most marked on the 2nd day of incubation. (Figs. 2, 3, 5)
- C (br) is very similar to C except that it is larger and has a thin smooth halo which is less turbid and therefore less obvious than the C halo. (Figs. 4, 5)
- T (m) (minute) is a small turbid plaque which appears to be merely the center of the T plaque with a sharply demarcated outside ring. It is very variable in size. Phages of this type were isolated both by recombination of C (m) and T and from a lysogenic strain as mentioned previously. (Figs. 1, 3)
- C (m) is a small, variable, extremely clear plaque with a sharply demarcated outer limit. In other words, it is haloless. (Figs. 1, 3, 4)
- T (vm) (very minute) produces plaques so small as to be barely visible. When plated on 7 per cent peptone agar, the virus produces larger plaques, very similar to T (m). The C analogue has not been studied but it is similar to C (m). T (vm) is much less stable when stored at 4°C. in 5 per cent peptone than the other viruses. (Not illustrated.)
- S (semiturbid) phage produced T-like plaques with clear centers. The study of this strain is not completed. (Not illustrated.)

For suitable differentiation two types of plates were used in this study. Most of the phage preparations were plated on standard Petri dishes containing 30 cc. of 5 per cent peptone agar, which produces medium sized plaques with fair differentiation. However, in differentiating C from C (br) and in counting the smaller phages (T (m); T (vm)), it is preferable to use plates containing 30 cc. of 7 per cent peptone agar. This gives excellent differentiation and very large plaques. It has the disadvantage of not being satisfactory for much more than 150 plaques per plate, while the 5 per cent peptone may be suitable for counts up to 700 or more.

Other Differences.—There is a definite difference between the way the C phages and the T phages react in peptone broth cultures. By using 5×10^7 *B. megatherium* (KM) cells to start with and three times that many C phage infective units, complete clearing is produced, followed, after 3 to 5 hours, by a regrowth of resistant cells. A high titer of bacteria (5×10^8) and phage (3×10^{10}) is present after 24 hours. The regrowth of cultures infected with C(m) virus appears at the same time but the growth rate is far slower.

The T group only partially lyses the cells and their regrowth begins almost immediately. After 24 hours the bacterial count is as high as the control (*B. megatherium* without phage), 10^9 , but the phage titer has fallen from $5-10 \times 10^9$, at the time of lysis, to 5×10^8 phage per cc. T(br) varies slightly in that it causes less lysis and a slower rate of regrowth. However, after several hours, it reaches the same level as the other T viruses. The regrowth in this group seems to be made up of lysogenic cells.

Recombination Experiments.—All possible combinations of pairs of these phage strains have been tested at least twice by the method described, except T(vm) and S. The percentages of the two original phages, the two recombinant types, and the mixed plaques are shown in Table I for the productive combinations. Mixed plaques are recognizable in all cultures containing a C and T virus and may best be described as a C with a T center. They always contain at least one T and one C type virus.

The only pairs which produced recognized new types were those in which a T of one minor type and a C of a different minor type were mixed. Furthermore, in all cases, the recombinants, when inoculated together, produced the originals.

TABLE I

Combination	Before or after lysis	Plaque count per bacterium	Percentages of types in phage population					
			Original phages		Recombinants		Large mixed	Small mixed
			C(m)	T	C	T(m)		
C(m)×T	Before	0.37	20	32	0	0	48	0.15
	After	120	38	55	3	4	0.3	
C×T(m)	Before	0.28	31	17	0	0	52	0.3
	After	94	66	26	3.5	3.5	0.4	
C(br)×T	Before	0.34	10	7	0	0	83	0.7
	After	300	59	35	2	3	0.7	
C×T(br)	Before	0.33	20	9	0	0	71	0.4
	After	170	61	33	3	3	0.4	
C(br)×T(m)	Before	0.20	21	17	0	0	62	0.1
	After	80	61	29	3.5	6.5	0.1	
C(m)×T(br)	Before	0.26	25	49	0	0	26	0.1
	After	190	16	80	2	1.5	0.3	

An effort was made to learn whether all the bacteria were infected with both original phages and released both original phage types. After the bacterial suspensions were exposed to the mixture of the two phages, 70 to 80 per cent of the phage activity was removed by centrifuging the bacteria. Since there was originally a 10 to 1 ratio of each phage per bacterial cell, this means that the average cell had received 7 to 9 phage particles of each type. However, the problem was greatly complicated by the fact that the majority of the bacteria were in short chains. A few single-chain burst studies (10) showed that both original and recombinant phages were produced by single chains. The

number of experiments necessary to indicate that the single cells produced recombinants was considered too formidable to be undertaken at this time. The absence of such data severely restricts any great emphasis being placed on the absolute percentages of recombinants shown in Table I.

However, the same percentages were found in experiments in which the bacterial and phage concentrations were varied over a wide range, provided there was at least a threefold majority of virus per bacterium. The observer's ability to make the differential counts has been tested by making known artificial mixtures. Although counts generally were within the usual experimental error, the subjective factor could not be ruled out and therefore, we recorded only plates which were technically excellent and replated many plaques which were doubtful.

Mixed Plaques.—It will be noted that there was a persistence of mixed plaques after lysis. A calculation of the absolute numbers of these forms shows that there was probably an actual increase in their number. However, the percentages involved were so small that the accuracy was not sufficient to prove this definitely. Furthermore, there was such a wide spread in the percentages found in individual experiments, that it is thought that all the mixed plaques have not been recognized in all cases. Mixed plaques were not due to overlapping since artificial mixtures of the four types plated at high plaque density have not produced these forms.

Before lysis, the mixed plaques tended to have a slightly more definite T component in them, while after lysis, the plaques appeared as C's with small, central, turbid zones. Furthermore, the small mixed type did not appear until after lysis, except in the non-productive combination $C(m) \times T(m)$. The large mixed plaques contained all four phages in the reaction or a homologous T and C of the larger minor types. The small mixed plaques yielded only $T(m)$ and $C(m)$.

There was a further difference between the mixed plaques before and after lysis. If the specimens were filtered through a half Seitz (E.K.) pad before lysis, no mixed plaques appeared when the filtrate was plated. This presumably indicates that the mixed plaques were being produced by mixedly infected bacteria. However, after lysis was complete, the number of mixed plaques was not influenced by the filtration. This indicates that these plaques were formed by particles which could pass through the filter. After an extensive study of a similar phenomenon in the *coli*-phage system, Hershey (11) has come to the conclusion that the mixed plaques are formed by single particles of "heterozygous" phage.

DISCUSSION

There is no apparent reaction between two members of the T group, nor two members of the C group. Furthermore, there is no apparent reaction

between a T of one minor type and its C analogue. However, if one were to predict from Hershey's (5) results with the r and m mutants of *coli*-phage, one would expect the reaction T(br) \times T(m) to produce the recombinants T (wild type) and T (br)(m). Persistent failure to demonstrate this type of reaction has led us to very careful search for it without success. One can conclude either that no such combinations are possible with these *B. megatherium* phages or that they occur in such low percentages as to be very difficult to find. At any rate, they must occur with a frequency well below 0.5 per cent. It may be that T(br) and T(m) could be considered as demonstrating multiple allelism, an example of which has been recently described for the *coli*-phage system by Hershey and Davidson (12).

The method by which these strains were isolated bears mentioning since it has been valuable in this work. Apparently it is not possible for the KM strain of *B. megatherium* to become C lysogenic (in either 5 per cent peptone or in asparagine media) and still produce colonies on 5 per cent peptone agar. On the other hand, lysogenic cells infected with a different strain of phage are not infected by C phage. Therefore, any lysogenic colonies derived from a culture of sensitive megatherium and phage C, must produce a different phage. This has been found to be the case. This is a far more efficient method of isolating new strains than examining hundreds of plaques for single variants.

An analysis of the mixed plaque is not possible with the present limited data. Hershey and Chase (11) have interpreted a similar phenomenon in the *coli*-phage system as evidence for heterozygosity. A far more thorough study of the *B. megatherium* system would be necessary before a like conclusion could be drawn, although marked similarities are present.

I wish to express my sincere appreciation to Dr. John H. Northrop for his interest and criticism.

SUMMARY

Repeated transfers in 5 per cent peptone or asparagine media of sensitive *B. megatherium* cultures containing C phage have made possible the isolation of lysogenic bacteria which produce different strains of phage. This is probably due to the failure of C virus to make the lysogenic adjustment while those of its variants which can succeed are selected.

Four distinct plaque types have been isolated by this method and some of their reactions in mixedly infected bacteria are described.

It has been shown that it is possible to isolate either C or T analogues of each of the strains, bringing the reported plaque types of *B. megatherium* phage to eight.

No success was obtained in an attempt to isolate a wild type and a phage carrying two mutations when crosses were made of two phages each differing by one mutational step.

BIBLIOGRAPHY

1. den Dooren de Jong, L. E., *Zentr. Bakt., 1. A pt., Orig.*, 1931, **120**, 1.
2. Gratia, A., *Compt. rend. Soc. biol.*, 1936, **123**, 1018.
3. Burnet, F. M., and Lush, D., *Australian J. Exp. Biol. and Med.*, 1936, **14**, 27.
4. Sertic, V., *Compt. rend. Soc. biol.*, 1929, **100**, 614.
5. Hershey, A. D., and Rotman, R., *Genetics*, 1949, **34**, 44.
6. Delbrück, M., and Bailey, W. T., Jr., *Cold Spring Harbor Symp. Quant. Biol.*, 1946, **11**, 33.
7. Hershey, A. D., *Cold Spring Harbor Symp. Quant. Biol.*, 1946, **11**, 67.
8. Gratia, A., *Ann. Inst. Pasteur*, 1936, **57**, 652.
9. Northrop, J. H., *J. Gen. Physiol.*, 1951, **34**, 715.
10. Burnet, F. M., *Brit. J. Exp. Path.*, 1929, **10**, 109.
11. Hershey, A. D., and Chase, M., *Cold Spring Harbor Symp. Quant. Biol.*, 1951, **16**, 471.
12. Hershey, A. D., and Davidson, H., *Genetics*, 1951, **36**, 667.

EXPLANATION OF PLATES

5 per cent peptone plates were used for Figs. 1 through 4.

PLATE 27

FIG. 1 *a*. The results of a combined infection with T and C(m) phage. It will be noted that besides the two originals, C(br) and T(br) are present. A "small mixed" plaque is also indicated (a). $\times 2.2$.

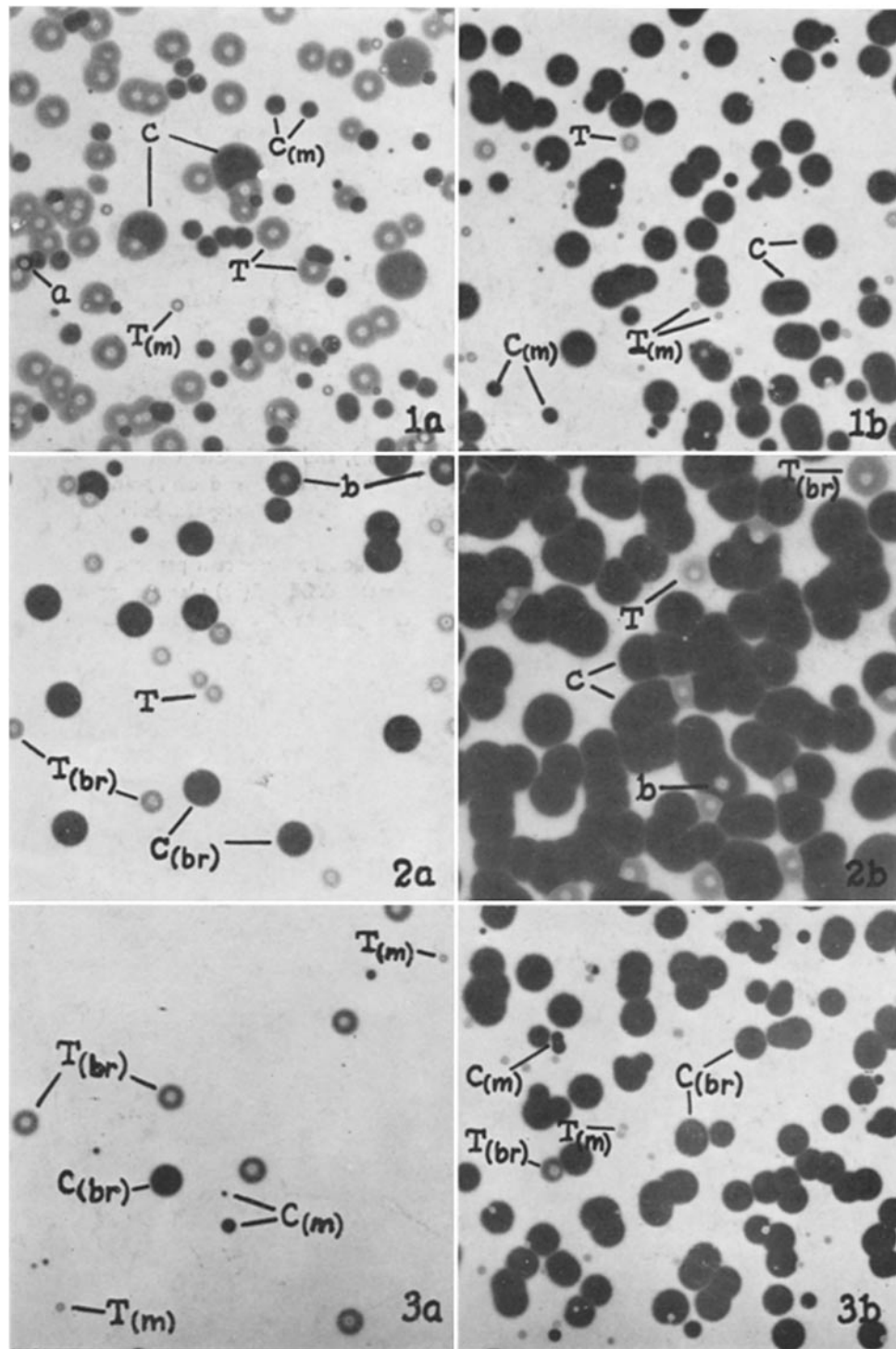
FIG. 1 *b*. The reverse reaction; C and T(m) have produced T and C(m). $\times 2.2$.

FIG. 2 *a*. The effect of combining C(br) and T. T(br) may be recognized, but the difference between C and C(br), both of which are present, is not sufficient for reproduction. (See Fig. 4.) Two "large mixed" plaques are apparent in the upper right (b). $\times 2.2$.

FIG. 2 *b*. The reverse of Fig. 2 *a*. Once more it is not possible to differentiate C and C(br). $\times 2.2$.

FIG. 3 *a*. The result of combining C(m) and T(br). The recombinants C(br) and T(m) appear.

FIG. 3 *b*. The reverse reaction. $\times 2.2$.

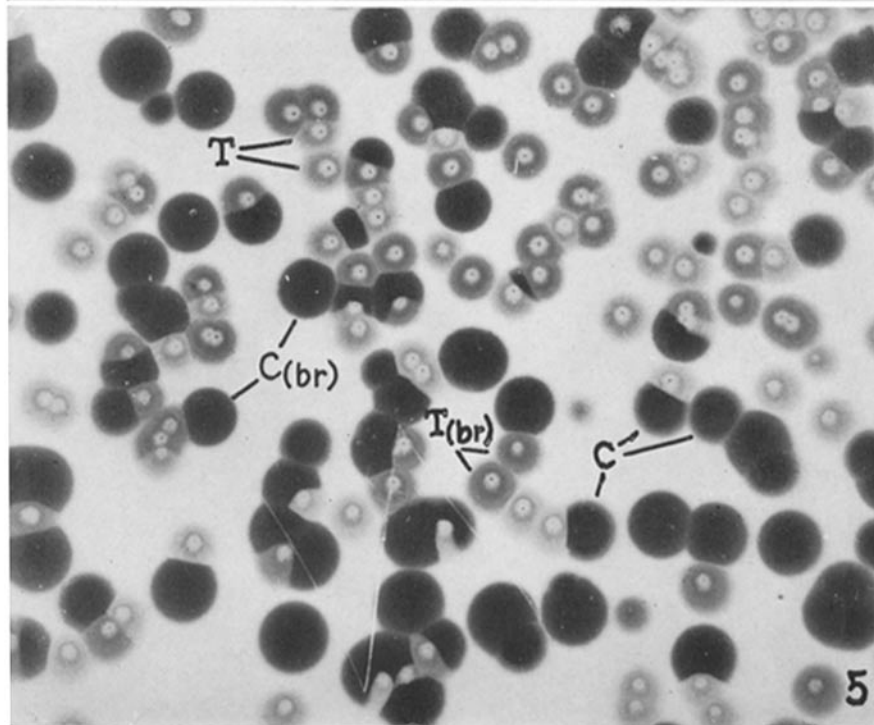
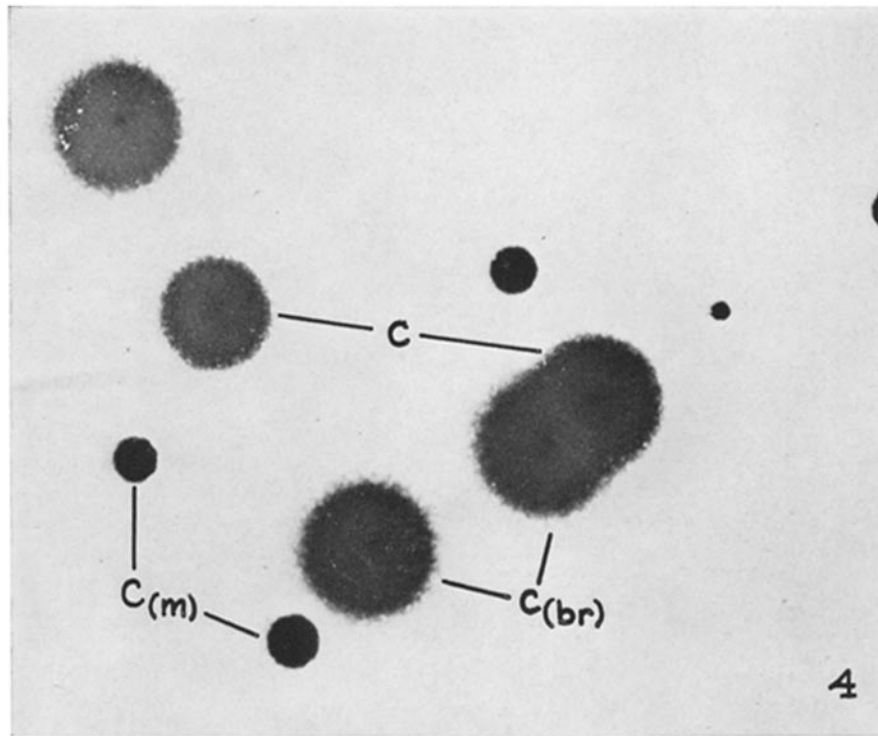


(Murphy: Mutants of bacteriophage of *Bacillus megatherium*)

PLATE 28

FIG. 4. Differences in the three C strains, C, C(br), and C(m). The three strains were plated together on a 5 per cent peptone agar plate and stained with safranine for the photograph. Note the absence of halo in C(m), the harsh stippled halo of C, and the soft halo of C(br). $\times 10$.

FIG. 5. Artificial mixture of C, C(br), T, T(br) made on a 7 per cent peptone agar plate. The contrast between T and T(br) is very marked. The C(br) plaques appear large and show a very faint halo. The C plaques are slightly smaller and show a definite halo. $\times 2.2$.



(Murphy: Mutants of bacteriophage of *Bacillus megatherium*)