# STUDIES ON LYSOGENESIS

## III. Superinfection of Lysogenic Shigella dysenteriae with Temperate Mutants of the Carried Phage[1]

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Bacteria able to produce a phage in absence of a recent infection are called lysogenic. When superinfected with that phage, they are immune to lysis. Each lysogenic cell (or nucleus, or other indispensable organ of a lysogenic cell) carries a fixed number of structures (=prophages; Lwoff and Gutmann, 1950) which reproduce, are regularly passed on to the daughter cells when the cell divides, and cause the carrier cell to be lysogenic. The experimental evidence supporting this statement has been presented elsewhere (Bertani, 1953*a*) together with a general discussion of the problem.

The experiments described in this paper represent a genetic approach to the problem of the prophage. Lysogenic cells were superinfected with phages that differed from the type of the prophage by one or more mutational steps, and the interactions taking place between the prophage and the superinfecting phage were analyzed.

### MATERIALS AND METHODS

*Bacteria.* The bacterial strains, *Sh* and *Sh/s* of *Shigella dysenteriae*, have been described before (Bertani, 1951). A number of lysogenic derivatives of *Sh* were isolated from the bacterial growth visible in the centers of the plaques formed by temperate phage *P2* and by its temperate mutants on *Sh*. Lysogenic strains are designated with the symbol for the bacterial strain followed by the symbol for the carried phage in parentheses: for example, *Sh*(*P2*). These strains do not differ from *Sh* in growth rate or in ability to adsorb phages. In the exponential phase of growth, spontaneous cell lysis with production of phage occurs at a rate of the order of $10^{-4}$ cells per generation. The burst size is around 100. The ratio (bacteria)/(free phage) varies between 50 and 200.

*Phages.* The general properties of temperate phage *P2* have been described by Bertani and Weigle (1953). Phage *P2* needs $Ca^{++}$ for attachment to the bacterial cells. If the adsorption mixture is diluted into medium with no $Ca^{++}$ added, a large proportion of the adsorbed phage may reappear as free phage in the medium. Except for minor quantitative differences, these properties are common to all mutants of phage *P2* which have been tested, both on strain *Sh* and on strain *Sh*(*P2*).

Several mutants of *P2* have been used. Wild type *P2*, plated on *Sh* or *Sh/s*, forms turbid plaques, 1 to 4 mm in size, with very irregular contour (figure 1a). In a *P2* preparation some plaques with more exactly round contour were once found. They bred true and were designated *P2 rd* (*rd* for *round*; figure 1b). *P2 rd* mutates to a larger type, also round, which was designated *P2 rd l* (*l* for *large*; figure 1c). *P2 l* (figure 1d) was obtained by recombination from *P2 rd l* and *P2*. In mixtures of different types, it is not always easy to distinguish *P2 l* from *P2*, and *P2 rd* from *P2 rd l*. This difficulty can be partially overcome by plating on a colored agar, CLB agar, described below. On this medium, *P2* and *P2 rd* are hardly distinguishable; both show a well defined yellowish halo. *P2 rd l* gives large, round plaques with a whitish halo, easily distinguishable from those of *P2* or *P2 rd*. *P2 l* plates with low efficiency, but its plaques are clearly distinct from the other types, being smaller and without halo.

Another plaque type, *P2 rd b* (*b* for *blue*) was obtained by mutation from *P2 rd*. Its plaques (figure 1e) are not very different from those of *P2 rd l* on regular nutrient agar; on CLB agar *P2 rd b* plaques show no halo at all and look blue, rather resembling *P2 l* plaques.

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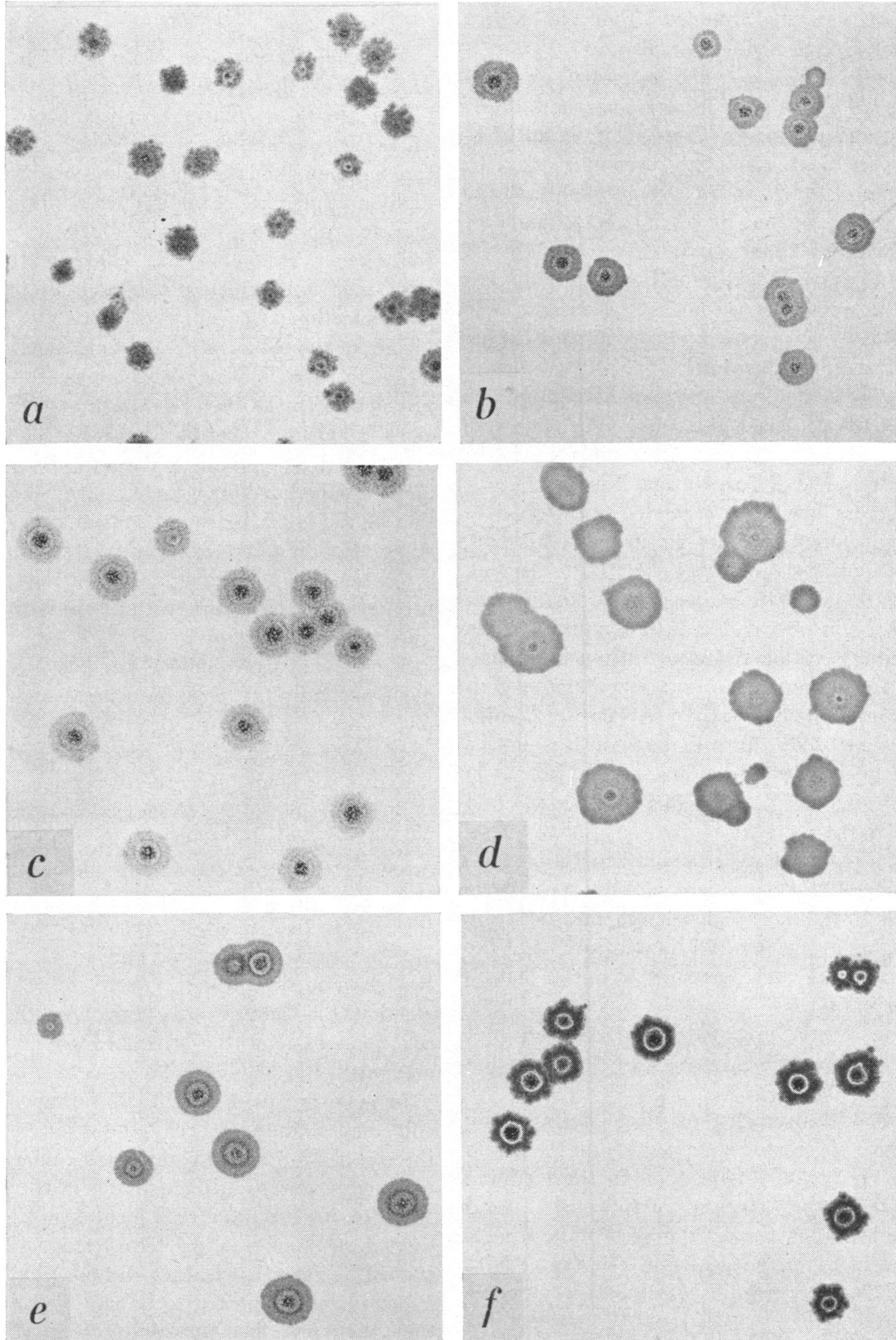


*Figure 1.* Plaque types of phage *P2* on its indicator *Shigella dysenteriae*, strain *Sh/s.* (a) *P2* (wild type); (b) *P2 rd;* (c) *P2 rd l;* (d) *P2 l;* (e) *P2 rd b;* (f) *P2 c.* Magnification 3.3 ×.

On regular nutrient agar all the mutants described above form turbid plaques from which lysogenic isolates, *Sh(P2 rd)*, *Sh(P2 l)*, etc., are easily obtained. A mutant giving clearer plaques, *P2 c* (*c* for *clear*), was also isolated (figure 1f). This mutation does not affect the shape of the plaque but only its degree of turbidity. The *c* mutant is temperate since lysogenic isolates,

*Sh(P2 c)*, can be obtained from the scarce secondary growth visible in *c* plaques.

The sign + is used, when necessary, to designate the wild type allele of any given mutation.

*Media and procedures.* The experimental techniques were those commonly used in work with the phages of the *T*- series (Adams, 1950). Phage lysates were prepared by the plate technique of Swanstrom and Adams (1951).

The LB nutrient medium (Bertani, 1951) was used. In certain experiments, CLB agar was used: this was prepared according to Bresch (1953) by adding glucose (1.5 per cent), "alizarol yellow GW" (0.062 per cent), and "soluble blue 3B Ex" (0.01 per cent) to LB agar. The dyes were obtained from National Aniline Division, Allied Chemical and Dye Corporation, New York.

Adsorption was always carried out with cells from an actively growing culture in LB, spun, and concentrated to about $2 \times 10^8$ per ml. $CaCl_2$ (0.005 to 0.01 M) was added to increase the adsorption rate, and at least the first dilution following adsorption was done into medium containing $Ca^{++}$ to avoid desorption. Some experiments were done before the possibility of desorption was realized; this will be indicated in the text. In some cases adsorption was carried out in presence of NaCN according to Benzer and Jacob (1953).

Adsorption times varied from 5 to 15 minutes, to obtain 80 to 95 per cent of the input phage adsorbed. Unadsorbed phage was measured by centrifuging at low speed a diluted sample of the infected culture and assaying the phage left in the supernatant.

Immune serum, prepared by injecting a rabbit with *P2* preparations, was used to neutralize unadsorbed phage.

To avoid readsorption of phage liberated by growing lysogenic bacteria, sodium citrate (0.5 per cent) was often added to the medium. Citrate traps $Ca^{++}$ present in the medium and thus reduces considerably the adsorption of *P2*.

The streptomycin technique (Bertani, 1951) was used to assay free phage present in lysogenic cultures.

To test lysogenic colonies for the type of phage carried, a small amount of LB medium was inoculated with each colony and incubated for several hours. A loopful from each culture was then spot tested for phage on *Sh/s* in the presence of streptomycin.

## RESULTS

*Superinfection in mass cultures.* When actively growing cells lysogenic for phage *P2* are superinfected with a temperate mutant of *P2*, the following facts are observed: (a) All cells survive the superinfection. Some cell loss observed with high multiplicities of superinfection probably is due to lysis from without (Delbrück, 1940). (b) The superinfected cells continue to divide with practically no lag at the same rate as before superinfection. (c) They continue to produce phage at the same rate as before superinfection although there may be a slight increase in phage production in the first two or three cell generations following superinfection. (d) The superinfected culture produces both the carried type and the superinfecting type of phage. The proportion of the latter reaches a maximum soon after superinfection and decreases to a minimum value a few generations thereafter. From then on, the proportion of the superinfecting type in the phage yield remains constant.

These points are demonstrated in the experiment of figure 2, where the amount and type of phage produced by strain *Sh(P2)* cells superinfected with phage *P2 rd l* were followed over a period of 12 hours. By 3 hours after superinfection, 18 per cent of the phage liberated was of the superinfecting, *rd l* type. By 6 hours this had dropped to 3.1 per cent and remained constant thereafter. As a consequence of superinfection the culture had acquired the ability to produce indefinitely a constant proportion of phage of the superinfecting type. Then it was tested, whether this new property involved the whole cell population of the superinfected culture or only part of it.

In the experiment of figure 2, the superinfected culture was diluted and plated out for colonies at the end of the 12 hour period during which phage production was studied. A sample of the colonies thus obtained was analyzed for the type of phage carried. Out of 128 colonies tested, 124 were typical *Sh(P2)*, that is, they did not produce any *P2 rd l*; three appeared to be like *Sh(P2 rd l)* in that they had lost the ability to produce *P2* and had become lysogenic for the superinfecting phage; one was able to produce both types of phage and was designated *Sh(P2)(P2 rd l)*. All these types bred true. For the *Sh(P2)(P2 rd l)* isolate, in particular, ten subcolonies were tested and all were able to produce both types of phages.

Thus, a small proportion of cells in the superinfected culture had been modified permanently: in some, a substitution of prophage type had occurred; others had become doubly lysogenic. Prophage substitution and double lysogenization account quantitatively for the final small but constant proportion of the superinfecting type in the phage produced by a superinfected culture.

*Prophage substitution and double lysogenization.* Most of the observed cases of prophage substitution and of double lysogenization, obtained in experiments similar to the one described above, are listed in table 1. (a) Prophage substitution was obtained with all combinations of carried and superinfecting phage that were tried. (b) There is a definite positive correlation between multiplicity of superinfection and frequency of prophage substitution. (c) Several stable doubly lysogenic isolates were found in these experiments. (d) In a number of instances colonies (called "unsegregated" in table 1) were found which appeared to liberate phage of both the prophage and the superinfecting types, but which upon replating gave rise to a mixture of substituted and unsubstituted lines, both types breeding true. Prophage substitution apparently had not been completed at the time the cells had been plated for colony testing, that is, as late as 16 cell generations after superinfection. The unsegregated colonies cannot originate by readsorption of free phage since they occur even when readsorption is blocked by addition of citrate to the medium during growth after superinfection. This finding remains unexplained for the time being. (e) Instances of genetic recombination between the carried phage and the superinfecting phage were observed. A colony of *Sh(P2 rd l c)* and one of *Sh(P2 + + +)* in experiment b of table 1, and one of *Sh(P2 + + +)* in experiment d were isolated. Besides, in experiment b a doubly lysogenic colony was found which showed segregation for two true breeding types: *Sh(P2 + + c)(P2 rd l +)* and *Sh(P2 + + +)(P2 rd l +)*. The latter type is also presumably the result of a recombination process.

The occurrence of genetic recombination in some instances of prophage substitution indicates that substitution does not necessarily involve the whole prophage. It is even conceivable that substitution is *always* the consequence of genetic recombination between prophage and superinfecting phage.

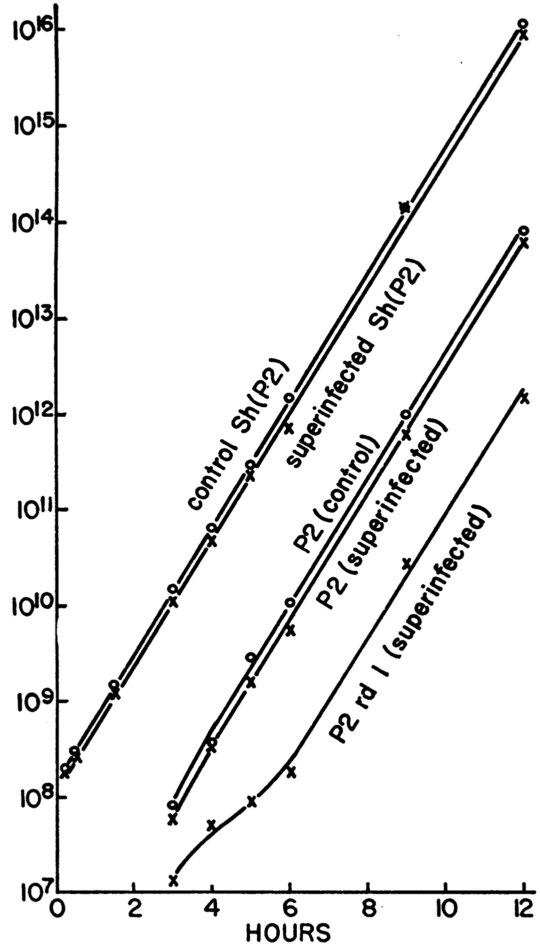


*Figure 2.* Bacterial growth and phage production in control and superinfected lysogenic cultures at 37 C. Strain *Shigella dysenteriae Sh(P2)* superinfected with *P2 rd l*. Adsorption (10 minutes) in LB with $CaCl_2$ ($2 \times 10^8$ cells and $3.3 \times 10^9$ phage per ml), followed by dilution into LB with anti-*P2* serum, and later into LB with citrate. Desorption was not controlled in this experiment, so that the actual multiplicity of superinfection may have been as low as 7 or 8. As the cells grew, new dilutions into the same medium were made to keep them continually in the exponential phase of growth. Titers are referred to the adsorption tube.

The general properties of double lysogenic strains have been briefly described before (Bertani, 1953*a*). A comment will be added here.

In the phage yield of a doubly lysogenic strain, recombinant types may be found. Thus, strain *Sh(P2 + +)(P2 rd l)* produces a small amount

TABLE 1

*Prophage substitution and double lysogenization*

Lysogenic cells were superinfected, diluted in LB medium, and incubated at 37 C with aeration for several hours. To avoid readsorption of the phage liberated, the bacterial titer was kept low by diluting into new medium at intervals during the incubation, and in some experiments (a, b, d, and e) citrate was added to the medium. Bacterial assays were made at the end of the incubation by spreading adequate dilutions on nutrient agar. The colonies were tested for type of phage carried. When more than one type was found, the colony was analyzed further by restreaking on agar and testing a number of subcolonies. If the original clone resulted to be a mixture of bacterial types, it was considered as unsegregated. No mixtures of more than two types were found. In the table, the columns for "substituted" and "doubly lysogenic" include the unsegregated and the recombinant colonies. The data from the experiment of figure 2 are not included in this table.

| EXPERIMENT | PHAGE TYPE | | MULTIPLICITY OF SUPERINFECTION | GENERATIONS ELAPSED BEFORE TESTING | TOTAL COLONIES TESTED | TOTAL SUBSTITUTED | PERCENTAGE SUBSTITUTED | TOTAL DOUBLE LYSOGENIC | UNSEGREGATED | RECOMBINANTS |
|---|---|---|---|---|---|---|---|---|---|---|
| | Carried | Superinfecting | | | | | | | | |
| a | *rd* + | + *l* | 12.2 | 15 | 194 | 22 | 11.3 | 3 | 1 | 0 |
| b | + + *c* | *rd l* + | 11.2 | 14 | 215 | 20 | 9.3 | 1 | 3 | 3 |
| c | *rd l* | + + | 11.1 | 10 | 261 | 7 | 2.7 | 0 | 1 | 0 |
| d | + + *c* | *rd l* + | 9.3 | 16 | 166 | 9 | 5.4 | 0 | 2 | 1 |
| e | *c* | + | 8.5 | 12 | 204 | 11 | 5.4 | 0 | 4 | |
| f | *rd* + *b* | *rd l* + | 5.8 | 7 | 236 | 6 | 2.5 | 0 | 2 | 0 |
| g | + + | *rd l* | 3.6 | 9 | 41 | 1 | 2.4 | 0 | 0 | 0 |
| h | + + | *rd l* | 1.8 | 9 | 294 | 2 | 0.68 | 1 | 0 | 0 |

of *P2* + *l* (and presumably also of *P2 rd* +, but this is difficult to score when mixed with the parental types). Strain *Sh*(*P2 rd* +)(*P2* + *l*) liberates small amounts of *P2 rd l* and *P2* + +. Out of an approximate total of 18,800 plaques scored on CLB agar, 9 were *P2 rd l*, giving a recombination frequency of about $10^{-3}$. In either strain the new phage types cannot be the product of spontaneous mutation at one locus since they were never observed with strains singly lysogenic for the parental phage types. The recombination frequency is higher between locus *c* and the two loci *rd* and *l*, taken together. With *Sh*(*P2* + + *c*)(*P2 rd l* +), one easily finds plaques of types *P2 rd l c* and *P2* + + +. Out of a total of 1,890 plaques, 14 were *P2* + + + (*P2 rd l c* is more difficult to score), giving a recombination frequency of 1.5 per cent. Genetic recombination between *rd* and *l* in doubly lysogenic bacteria is, therefore, much less frequent than recombination between these two loci, taken together, and locus *c*. This is consistent with the failure to observe recombination between *rd* and *l* in the experiments on prophage substitution (table 1).

*The fate of the superinfecting phage.* The results of the experiments of figure 2 indicate that following superinfection some phage of the superinfecting type is produced independently of prophage substitution or double lysogenization. In fact, in the first four or five hours after superinfection more phage of the superinfecting type is liberated than can be accounted for by prophage substitution and double lysogenization. Later this excess becomes too small to be measured in the presence of the exponentially increasing phage of the superinfecting type yielded by the cells that underwent prophage substitution or double lysogenization. Conversely, it is likely that only a negligible proportion of the phage of the superinfecting type produced early after superinfection originates from cells that underwent prophage substitution, or double lysogenization.

The results of a modified single burst experiment (Bertani, 1951) also support this view. Cells of strain *Sh*(*P2*), superinfected with *P2 rd l* at high multiplicity, were distributed in small amounts into numerous tubes and incubated at 37 C for 3 hours, after which the content of each tube was plated for free phage. Out of 71 tubes, 36 had no phage, 18 had only *P2* phage, and 17 had both *P2* and *P2 rd l* phage. Assuming a random distribution of the phage yielding cells among the tubes, only 6 tubes are expected to contain phage derived from more than one cell; thus, most of the mixed phage yields come from

single cells. The average proportion of the *rd l* type in tubes containing both types was 30 per cent. The cells that produced phage of the superinfecting type during the first few generations following superinfection were thus neither cells that had undergone prophage substitution (which are expected to produce at lysis phage of only one type) nor cells that had become doubly lysogenic (which typically produce phages of the two carried types in average ratio 1:1; Bertani, 1953*a*).

We conclude that the superinfected cells transmitted to some of their progeny a transient ability to liberate some phage of the superinfecting type. The data can be best interpreted by assuming that a superinfecting phage particle may be carried without multiplying for a number of cell generations and may contribute genetically to the phage yield whenever the carrier cell happens to enter the lytic cycle.

There is evidence that the superinfecting phages are not carried over as externally adsorbed particles, that is, attached to the surface of the cells and otherwise intact. If it were so, detachment of superinfecting phage particles soon after adsorption should reduce the yield of phage of the superinfecting type in the superinfected culture. Lysogenic cells, *Sh*(*P2*), were superinfected with *P2 rd l*, left at 37 C for 15 minutes, and then treated for 10 minutes in a Waring blendor. This treatment, first used by Hershey and Chase (1952) to detach particles of phage *T2* adsorbed to the host cell surface, had been found to be applicable to phage *P2* adsorbed onto *Sh* (S. S. Lederberg, *personal communication*). The viability of the bacteria was not affected appreciably by the treatment. The same proportions of phage of the superinfecting type were observed, several generations after superinfection, in the phage yields of treated and untreated superinfected cells. This indicates that by the time of the blending the infection had proceeded beyond the mere attachment of the superinfecting particles to the surface of the cells.

We shall call *preprophage* the state assumed by the superinfecting phage following adsorption onto lysogenic cells. The preprophage participates in the phage yield of carrier cells that lyse spontaneously. It may become prophage, in which case it either substitutes for the prophage preexisting in the carrier cell or coexists with this prophage to establish the doubly lysogenic condition. We already mentioned that after a certain number of generations following superinfection all the phage of the superinfecting type is accounted for by doubly lysogenic or prophage-substituted cells. This indicates that preprophages do not multiply as such, but are progressively diluted out among the cell progeny of the originally superinfected cells. In the Appendix an attempt has been made to predict the proportion of cells carrying one or more preprophages at any given generation after superinfection, on the assumption that preprophages do not multiply and are randomly distributed to the daughter cells at cell division. A series of single burst experiments with cultures superinfected with various multiplicities of phage gave results that agree quite well with the expectations.

*Estimation of the number of prophages.* We may estimate the average number of prophages per lysogenic cell if we accept the interpretation proposed above and further assume that the relative amounts of phage of the superinfecting and of the carried type liberated by cells yielding both types are respectively proportional to the numbers of preprophages and of prophages present in the cells when the lytic multiplication of phage started.

In single burst experiments following superinfection with low multiplicities of phage, all the cells that give a mixed yield of phage contain, in first approximation, only one preprophage. The number of prophages per cell is given then by $(1 - x)/x$ where $x$ is the proportion of phage of the superinfecting type in the phage yields of such cells.

In a single burst experiment (similar to the one described in the preceding section, except for the lower multiplicity of superinfection of about 1), out of a total of 140 tubes, 40 had only phage of the carried type while 12 had phage of both types. The average value of $x$ for these 12 tubes was 0.31, giving 2.2 prophages per cell. Since *Shigella* cells, like other rod shaped bacteria, have usually from one to four nuclei per cell, the estimate obtained for the number of prophages per cell is in reasonable agreement with the hypothesis that (singly) lysogenic cells carry one prophage in each nucleus.

## DISCUSSION

The interpretation proposed above accounts for the results of experiments of superinfection of lysogenic bacteria with temperate phages closely

related to the carried phage. The theory is supported by superinfection experiments done by Jacob and Wollman (*personal communication*) with phage *lambda*, which may be induced (Lwoff, 1953) by ultraviolet irradiation, thus permitting an easy determination of the proportion of mixed bursts at any given time after superinfection. Jacob and Wollman (1953) also estimated the prophage number, using assumptions similar to ours, with a different material and obtained results consistent with ours.

The theory cannot yet be extended to explain the results of superinfection experiments with weak virulent phages related to the carried phage (Bertani, 1953*b*).

The preprophage differs from both vegetative phage (Hershey, 1953; Visconti, 1953) and prophage insofar as it does not multiply as such, and insofar as its presence does not necessarily lead to lysis. But a preprophage may become a vegetative phage (in those cells that are going to liberate phage) or may become a prophage (in prophage substitution and double lysogenization). Thus, it can be considered as a state in the developmental cycle of phage, transiently blocked before the stage at which an irreversible decision for lysis or lysogenization is taken (*see* Bertani, 1953*a*).

Stages similar or identical to the preprophage probably occur in the process of lysogenization following infection of sensitive cells by temperate phages. Lieb (1953) concludes that this process consists of at least two steps: (a) infection of the cell without lytic multiplication of the phage; (b) attachment to the prophage site. Between the two steps several cell generations may occur, and the potentiality (preprophage?) to become lysogenic is segregated among the cell progeny. The main difference from the preprophage as defined from our experiments seems to be the fact that attachment to the prophage site occurs much more frequently than both prophage substitution and double lysogenization. It may very well be that in superinfection the prophage, on the one hand, blocks any immediate possibility of lytic development of the superinfecting phage, compelling it to remain at the preprophage stage; and on the other hand, by occupying the prophage site, reduces the chances of stable attachment of the preprophage. If this were correct, the difference between Lieb's infecting preprophage and our superinfecting preprophage would be only apparent.

It was shown by Hershey and Chase (1952), working with the virulent phage *T2*, that only the nucleic acid containing moiety of the phage enters the infected cell and is necessary for the multiplication of the phage. If these results can be generalized to apply to phage *P2*, the preprophage can be visualized as consisting of all or part of the nucleic acid of the superinfecting phage particle. Our experiments do not exclude the possibility that the superinfecting phage particle splits into a number of subunits (corresponding to linkage groups, for instance), each segregating independently of the others. This possibility could be tested by single burst experiments following superinfection with phage carrying two markers that give a high recombination frequency. Such markers are not yet available. More information on the genetics of phage *P2* should be helpful also in analyzing further the instances of recombination observed in doubly lysogenic strains and in prophage substitution.

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SUMMARY

Lysogenic cells were superinfected with temperate mutants of the carried phage. No lysis or killing of the superinfected cells was observed. The type of phage produced after superinfection was studied both in mass culture and in single burst experiments. Mixed liberation of the two phage types, the carried and the superinfecting one, occurred with a frequency decreasing with time after superinfection. The concept of *preprophage* is introduced; this is a developmental stage of the superinfecting phage, which does not multiply as such, is passed on to either daughter cell at cell division, and participates in the phage yield when the cell that carries it shifts into the lytic cycle. The progeny of superinfected cells was examined for any permanent modification produced by the superinfection. In a small proportion of cells the carried phage disappeared, being substituted either by the superinfecting

type or by a new type that was a genetic recombinant of the two. A still smaller proportion of cells became lysogenic for both the previously carried type and the superinfecting one. A method for estimating the average number of prophages in a lysogenic cell is indicated: the estimate obtained is consistent with the hypothesis that one prophage is present in each nucleus of a (singly) lysogenic cell.

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## APPENDIX

*Quantitative expectations from the definition of preprophage*. A temperate phage, upon superinfecting a bacterium lysogenic for a closely related phage, is postulated to assume the state of preprophage, which is defined as follows: (a) the preprophages do not multiply as such; (b) when a cell that carries one or more preprophages divides, the preprophages are distributed at random between the daughter cells; (c) when a lysogenic cell that carries one or more preprophages lyses spontaneously and produces phage, the preprophages participate with the prophage in the production of phage; (d) each preprophage has a small probability of becoming prophage, either by replacing the prophage or one of the prophages of the cell that carries it (prophage substitution), or by joining the existing prophage or prophages (double lysogenization). As a consequence of properties (a) and (b), the concentration of preprophages in an exponentially growing population of superinfected lysogenic cells must decrease exponentially. If prophage substitution, double lysogenization, and spontaneous lysis of lysogenic cells are rare events, they will not affect appreciably the rate of decrease of the concentration of preprophages in a growing cell population over a small number of generations following superinfection. As a consequence of property (c) lysogenic cells that carry one or more preprophages will always produce mixed yields of phage, containing, that is, both phage with the genetic markers of the superinfecting phage and phage with the markers of the carried phage.

These properties of the preprophage explain qualitatively the results of the superinfection experiments described in the text. The theory was tested quantitatively with single burst ex-

periments, in which the proportions of cells giving mixed phage yields during a certain period of time after superinfection with various multiplicities of phage were determined. The proportions expected were calculated as shown below, on the basis of assumptions (a) and (b). It was assumed further that no appreciable decay or elimination of the preprophages would occur during the few generations covered by the experiments.

If $N_o$ is the number of lysogenic cells at the time of superinfection and $p_o$ the average number of preprophages per cell immediately after superinfection (equal, therefore, to the multiplicity of superinfection), then, after $n$ generations:

$$N_n = N_o 2^n \quad (1)$$

and

$$p_n = p_o/2^n, \quad (2)$$

where $N_n$ and $p_n$ are, respectively, the number of cells and the average number of preprophages per cell after $n$ generations. The number of superinfecting phage particles per cell is assumed to follow a Poisson distribution. This assumption is satisfactory for low multiplicities of infection (Dulbecco, 1949). It can be demonstrated that the distribution of preprophages will remain Poisson also in the following cell generations. Therefore, at generation $n$, the proportion of cells carrying no preprophages will be given by $e^{-p_n}$. Assuming that the proportion of cells that lyse spontaneously during the growth of a lysogenic culture is directly proportional to the total number of "cells × generations", expressed as

$$\int_{x=o}^{x=n} N_x\,dx = \frac{N_n - N_o}{\log_e 2}, \quad (3)$$

one can then predict the *frequency of cells that lysed, liberating phage of the carried type only*, that is, the fraction of lysed cells that did not carry any preprophage:

$$A_{n,p_o} = \frac{\int_{x=o}^{x=n} N_x e^{-p_x}\,dx}{\int_{x=o}^{x=n} N_x\,dx} \quad (4)$$

$$= \frac{\log_e 2}{2^n - 1}\int_{x=o}^{x=n} 2^x e^{-p_o/2^x}\,dx.$$

The solution of (4) was given to us by Dr. E. S. Lennox as:

$$A_{n,p^o} = \frac{-p_o}{2^n - 1}\left[\frac{e^{-p_o}}{p_o} - Ei\left(-\frac{p_o}{2^n}\right) + Ei(-p_o) - \frac{e^{-p_o/2^n}}{(p_o/2^n)}\right] \quad (5)$$

where $-Ei(-x)$ is a tabulated function (W. P. A. tables, 1940).

*Single burst experiments to test the preprophage theory.* In these experiments lysogenic cells were superinfected with various multiplicities of phage, diluted, and distributed into a large number of small tubes in 0.5 ml aliquots. These tubes were incubated for several hours at 37 C, then chilled. A drop of streptomycin (about 250 $\mu$g) was added to each tube, and finally the whole content of each tube was plated on one plate in the presence of indicator bacteria *Sh/s*. Dilutions and incubation time were calculated aiming at obtaining, on the average, one cell lysing spontaneously for every 3 to 5 tubes. Unadsorbed phage was eliminated (although not completely) by adding serum after superinfection, centrifuging the serum treated cells at least once, and resuspending them in fresh medium. The time spent in centrifuging was not counted as incubation time as these operations were performed in a cold room.

The results of these experiments are summarized in table 2 and appear to support the theory. There is a good correlation between expected values ($A$) and experimental estimates of the proportions of phage yields from single cells (=bursts) containing phage of the carried type only (last two columns in table 2). A comparison of expected and observed values was made by the $\chi^2$ method. Of 8 independent estimates, only one was significantly different from its expected value. A possible cause for this discrepancy will be mentioned below, together with other comments necessary for an adequate evaluation of these results.

(a) Since some of the sensitive cells infected with a temperate phage become lysogenic rather than lysing, plaque counts may give low estimates of the titers of preparations of temperate phages when lysogenization occurs in a sizable fraction of the infected cells. In preliminary experiments we estimated that approximately 25 per cent of *Sh* cells become lysogenic upon infection with either *P2* or *P2 rd l* in liquid culture. If lysogeniza-

## TABLE 2

*Single burst experiments after superinfection*

Duplicate bacterial assays were done before superinfection. The same preparation of phage *P2 rd l* was used in experiments I, II, and III. Duplicate bacterial assays also were done before distribution into tubes, to calculate the bacterial inoculum per tube, $N_o$. At the end of the incubation period bacterial assays were obtained from six tubes in each series. From the average of these assays ($N_n$) and from $N_o$, the number of generations ($n$) elapsed during the incubation period was calculated. The average burst size was evaluated by dividing the total plaque count obtained from all plates with phage, by the number of bursts, calculated with the Poisson formula from the proportion of plates with no plaques. The burst frequency is expressed per cell generation, calculated from formula (3) in the text. Observed classes b and c may contain, of course, tubes with more than one burst. This has been taken into account in calculating the expected values for b and c from $A$ (equation (4) in the text), using the equation $A = 1 - \left(\log_e \frac{a-c}{a} \Big/ \log_e \frac{a-b-c}{a}\right)$, which is based on the assumption that the bursts of any given type are distributed among the tubes according to Poisson. The inverse calculation has been applied to obtain the observed values of $A$ from b and c.

| | Experiment | Multiplicity of superinfection, $p_0$ | Generations, $n$ | Generation time | Average burst size | Burst frequency (per cell generation), $\times 10^4$ | Tubes plated for phage | Tubes with phage of the carried type only | | Tubes with phage of the superinfecting type only or of both types | | $\chi^2$ comparing b and c with expected values | Frequency of bursts with phage of the carried type only, among all bursts | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Observed | Expected | Observed | Expected | | Observed | Expected |
| | | | | | | | a | b | | c | | | | A |
| | | | | min | | | | | | | | | | |
| *Sh(P2)* superinfected with *P2 rd l* | I | 3.4 | 3.6 | 41 | 79 | 1.1 | 130 | 7 | 10.7 | 14 | 10.3 | 2.61; 0.2>P>0.1 | 0.35 | 0.531 |
| Adsorption in cyanide | II | 4.8 | 3.6 | 41 | 87 | 1.6 | 132 | 17 | 19.9 | 36 | 33.1 | 0.68; 0.5>P>0.3 | 0.38 | 0.437 |
| | | 2.4 | 3.6 | 42 | 90 | 1.1 | 140 | 22 | 26.1 | 23 | 18.9 | 1.53; 0.3>P>0.2 | 0.54 | 0.626 |
| | | 1.2 | 3.2 | 48 | 67 | 1.7 | 140 | 40 | 36.5 | 13 | 16.5 | 1.08; 0.3>P>0.2 | 0.80 | 0.737 |
| Phage plating on LB agar | III | 3.7 | 2.9 | 55 | 80 | 3.3 | 137 | 31 | 19.3 | 32 | 43.7 | 10.2; 0.01>P>0.001 | 0.57 | 0.376 |
| | | 1.9 | 2.6 | 62 | 108 | 2.0 | 136 | 14 | 16.2 | 18 | 15.8 | 0.60; 0.5>P>0.3 | 0.47 | 0.538 |
| | | 0.94 | 2.9 | 55 | 129 | 1.7 | 140 | 26 | 26.5 | 10 | 9.5 | 0.36; 0.7>P>0.5 | 0.75 | 0.764 |
| *Sh(P2 rd b)* superinfected with *P2*. Adsorption in broth. Phage plating on CLB agar | IV* | 0.32 | 5.6 | 29 | | 1.2† | 69 | 56 | 54.0† | 13 | 15.0† | 0.34; 0.7>P>0.5 | 0.979† | 0.975 |

* In experiment IV, designed to test a low multiplicity of superinfection, the frequency of cells lysing per tube was kept much higher than in other experiments, taking advantage of the fact that on CLB agar plates plaques of *P2* can be scored easily in the presence of a large number of *P2 rd b* plaques.

† As a consequence of the different design of experiment IV, these values were calculated without making use of the Poisson formula as far as the total number of bursts in the experiment was concerned. This number was calculated from the estimated total number of plaques of all types (881 per tube) divided by the average burst size (91) which had been estimated in a previous independent experiment.

tion occurs with the same frequency under the conditions of plating, titration of these phages by plaque counts gives only 75 per cent of the phage particles plated. In absence of better information, a correction was introduced in all phage titrations involved in the experiments of table 2 by increasing the plaque counts by ⅓.

(b) It is difficult to eliminate completely the unadsorbed phage or the small amount of phage that may desorb after the end of the serum treatment (residual free phage). Since the expected frequency of bursts containing phage of the superinfecting type decreases exponentially as the number of generations after superinfection increases, the growth period after superinfection must not be too long, and therefore the inoculum per tube cannot be kept too low to avoid the use of huge numbers of tubes in each experiment. As a consequence, one cannot overcome the presence of residual free phage by simply diluting the inoculum further. Since the distribution of residual free phage in the tubes is expected to be of Poisson type, a correction was introduced in most experiments: tubes with *only one or two* particles of the superinfecting type were not counted in class c of table 2. This procedure is quite safe as long as such tubes did not contain phage of the carried type since bursts of one or two particles are of doubtful existence. It is less satisfactory for tubes containing both types of phage, the uncertainty depending on the shape of the frequency distribution of particles of the superinfecting type in mixed bursts. The number of tubes of this second class (containing both types of phage, the superinfecting one being represented by one or two plaques) in the experiments of table 2, listed in order, were 0 in experiment I; 1, 4, and 6 in II; 17, 5, and 9 in III; and 14 in IV. The cause of such a high level of residual free phage in experiment III is unknown. The level of residual free phage in experiment IV is also high, but this is expected since its different design required a larger bacterial inoculum per tube than was used in the other experiments.

(c) A small proportion of the tubes in which bursts had occurred (12 out of 303) contained only phage of the superinfecting type. In table 2, they were all grouped in class c. Theoretically, bursts of this type may be the consequence of any of three events. First, the population of lysogenic cells may contain some cells that have lost the prophage and reverted to the sensitive condition. These, when infected, would liberate a homogenous burst of phage of the type used in the infection. We know little about such reversions except that they are very rare and that they ought to be clonally distributed among different cultures. Second, cells of clones in which prophage substitution has occurred may lyse and therefore produce only phage of the superinfecting type. Third, homogenous bursts of phage of the superinfecting type may simply be the result of statistical fluctuations in burst size acting on a potentially mixed burst. The 12 tubes with phage of the superinfecting type only were distributed as follows: 5 in experiment I; 2, 1, and 1 in experiment II; and 1, 0, and 2 in experiment III. It does not seem necessary to introduce any correction in experiments II and III, especially since in these cases the number of plaques in such tubes tended to be smaller than the average burst size, indicating that statistical fluctuations may be the cause. In experiment I either a fraction of sensitive cells was present or prophage substitution occurred more often than usual. However, when a correction is applied here, either by transferring the 5 tubes in question from class c to class b, or by classifying them as tubes with no phage, the fit with the expected values is improved in either case.

(d) Considering that the main uncertainty comes from the correction of the titers of temperate phages (and therefore of the multiplicity of superinfection, $p_o$) for lysogenizing particles, rather than from statistical fluctuations in assaying phage or bacteria, the variance of $A$, equation (4), was not calculated, and in the calculation of $\chi^2$ the expected values were considered exact.

(e) No special control experiments were done to test whether superinfected populations of lysogenic cells show a higher frequency of spontaneous lysis with phage production than corresponding nonsuperinfected cells. The spontaneous burst frequency for *Sh(P2)*, as evaluated in 10 single burst experiments done for other purposes with slightly different techniques, varied from $2.4 \times 10^{-5}$ to $2.3 \times 10^{-4}$ per cell generation, the average being $7.0 \times 10^{-5}$. Since the average burst frequency after superinfection is $1.7 \times 10^{-4}$ (see table 2), it seems likely that the burst rate is slightly higher than normal for several

cell generations following superinfection. The available data are, however, insufficient for a quantitative analysis.

(f) It has been pointed out above that an estimate in experiment III of table 2 differed significantly from its expected value. A deviation in the observed direction could be expected if the variability of cell sizes in the culture used in experiment III were larger than usual, and this effect would be more obvious at higher multiplicities of infection (Dulbecco, 1949). The culture used in experiment III might have contained some filamentous cells which are often formed when the growth conditions are not optimal (an unfavorable batch of medium, for instance). The long generation times and the high average burst sizes observed in that experiment (see table 2) agree with this suggestion.