THE TRUE DIVISION AND DEATH RATES OF *SALMONELLA TYPHIMURIUM* IN THE MOUSE SPLEEN DETERMINED WITH SUPERINFECTING PHAGE P22

JOAN MAW AND G. G. MEYNELL

From the Guinness-Lister Research Unit, Lister Institute of Preventive Medicine, Chelsea Bridge Road, London, S.W.1

Received for publication 12 June, 1968

THE process inherent in infection is the multiplication of a parasite within a host. The rate at which a parasite succeeds in dividing *in vivo* is therefore a fundamental characteristic of a host-pathogen system, yet, for technical reasons, it has rarely been measured. Counting the number of viable organisms by conventional methods, like colony counts, can provide only a minimum estimate of the true division rate, since, in the absence of strong evidence to the contrary, the organisms must be presumed to be dying as well as dividing *in vivo*. Thus, if the true rates of microbial division and death are l and m respectively, viable counts necessarily measure l–m, the net rate of change, and not the individual rates (Meynell, 1959).

In general, l can be estimated in infected animals by determining how the numbers of dead, of living, or of dead and living organisms change with the passage of time. Measurement of the number of dead organisms alone is technically impossible, as far as is known. Measuring changes in the total population of organisms, both dead and alive, could be done by total counts (Rees and Hart, 1961) or by isotopic labelling. However, such an approach is inherently very insensitive because, when only a small fraction of the population is viable, its division will not cause any detectable alteration in the composition of the population as a whole (Meynell and Subbaiah, 1963). This drawback is overcome by measuring changes in only the viable fraction of the infecting population. To do this, a non-replicating genetic fragment is introduced into the cells and its subsequent segregation during cell multiplication *in vivo* followed. The method is applicable in principle to any kind of parasitic cell, including protozoa or animal cells, but with bacteria, the genetic fragment may be either a superinfecting phage genome or a piece of the bacterial genome transferred by abortive transduction (Meynell, 1959; Meynell and Subbaiah, 1963). All such methods measure l alone but, by using conventional viable counts to estimate l–m, the value of m is obtained by difference. Superinfecting phage rather than abortive transduction has been used here because it is considerably more sensitive.

In the superinfecting phage method, the bacteria to be inoculated are lysogenized by a temperate phage. Before inoculation into their host, they are superinfected with a differentially marked mutant of their prophage. The superinfecting phage neither lysogenizes the great majority of the bacteria nor replicates during their subsequent multiplication (Fig. 1). Thus, the proportion of bacteria carrying the superinfecting phage decreases in each bacterial generation in a predictable manner, as shown in Fig. 2. The curve initially has a shoulder

when the mean number of superinfecting phage per bacterium is appreciably greater than 1 and then falls exponentially, with the proportion of superinfected cells halving in each generation, once the majority of cells each contain only 1 superinfecting particle. The proportion of superinfected bacteria therefore bears a known relationship to the number of generations of growth. Moreover, the same relationship should hold *in vivo* even when divisions and deaths occur simultaneously, provided there is no selection either for or against cells carrying the superinfecting phage genome. Suppose, at a given moment, there are 10^6 viable cells of which 10^4 carry the superinfecting phage, and that 90 per cent

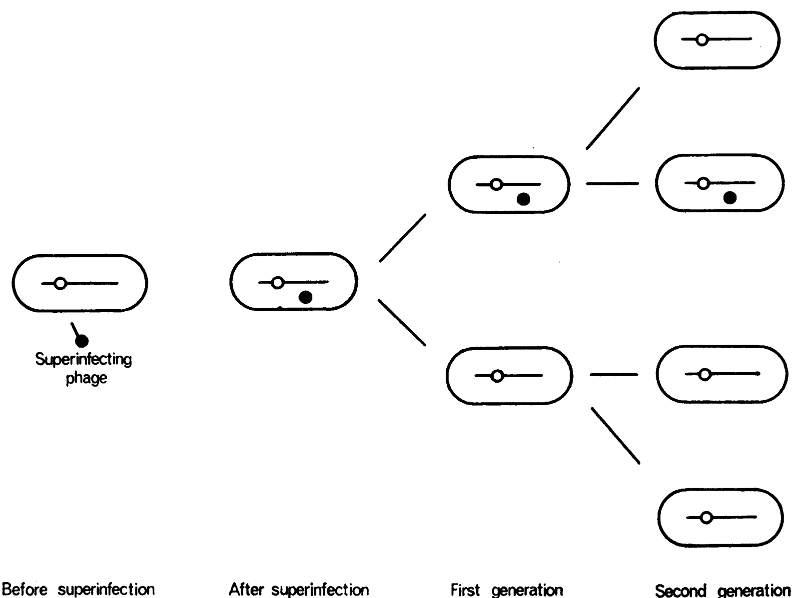


FIG. 1.—The principle of the superinfecting phage technique. Each bacterium carries the prophage, indicated by an open circle in the bacterial chromosome. The genome of the superinfecting phage, shown as a solid point, enters the lysogenic cell but cannot replicate. Hence, the proportion of cells carrying the superinfecting phage genome decreases in each bacterial generation.

of all cells then die: the proportion of superinfected cells is initially $10^4/10^6 = 0{\cdot}01$, and finally, $10^3/10^5 = 0{\cdot}01$, that is, the proportion remains 0·01 despite killing.

In practice, therefore, the mean number of generations that elapse *in vivo* in a given time is obtained, first, by inoculating the hosts with bacteria of which a known proportion carry the superinfecting phage and, second, by recovering the bacteria from the host after a known time and again measuring the proportion. The proportion of superinfected cells is determined by inducing vegetative phage growth by exposure to U.V. radiation, followed by plating before bacterial lysis occurs on bacterial indicator strains specific for the prophage and the superinfecting phage, respectively. The proportion is then the ratio of the plaque counts on the two indicators. Given a curve like that of Fig. 2, constructed for each experiment (see Methods), the number of generations elapsing between inoculation and postmortem is obtained directly.

The method was originally used with *Escherichia coli* (Meynell, 1959) and has now been successfully extended to salmonella infections. Its applicability clearly depends on survival of the superinfecting genome within the bacteria. This is observed with certain wild type phages like P2 (Bertani, 1954) and λ (Jacob, 1954), but, unfortunately, their bacterial hosts are not pathogenic for mice. On the other hand, *Salmonella typhimurium* is a well known mouse pathogen but the

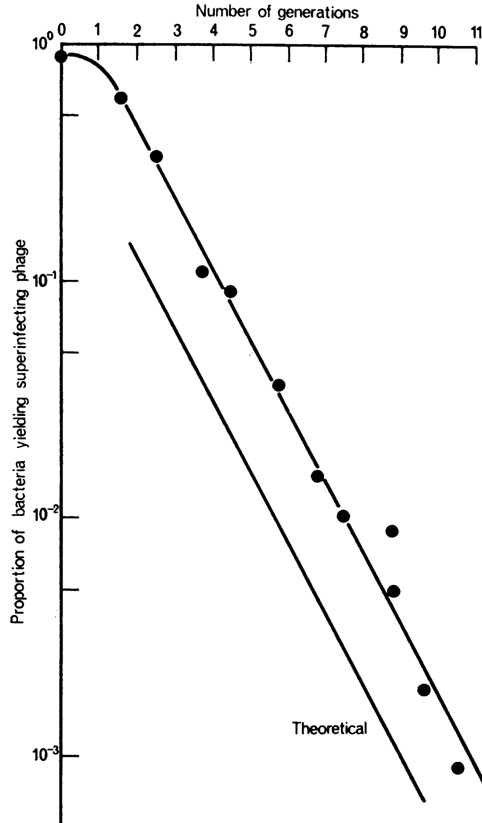


FIG. 2.—The behaviour of superinfecting phage during bacterial growth in broth. The scale of the abscissa differs in the two parts of the Fig. During the first 10 generations after superinfection, the phage dilutes out as expected. The theoretical curve represents a halving of the proportion of superinfected organisms in each generation (Fig. 2a). Following the 10th generation, the curve ceases to decrease exponentially following the outgrowth of that proportion of bacteria in which the superinfecting phage genome has become integrated (FIG. 2b). This proportion was assumed to be 5×10^{-6} in fitting the calculated curve to the Fig. 2b points.

best studied salmonella phage, P22, is not recovered after superinfection from strains carrying it as prophage (Walsh and Meynell, 1967*a*). Wild type phage P22 therefore excludes superinfecting P22 and is termed " excluding " ($x+$) but we have recently isolated non-excluding (x) mutants (Walsh and Meynell, 1967*a*). Strains lysogenized by x mutant phage permit recovery of superinfecting phage P22, although remaining immune to superinfection, because the immunity conferred by the P22 prophage depends not only on exclusion of the superinfecting

phage but also upon repression of its vegetative growth. Using this phage, the method was applied to *Salm. typhimurium* growing in the spleens of mice infected by intravenous inoculation and shows that the true division rate *in vivo* corresponds to a doubling time of 5–12 hr. compared to a minimum of 0·5 hr. in broth.

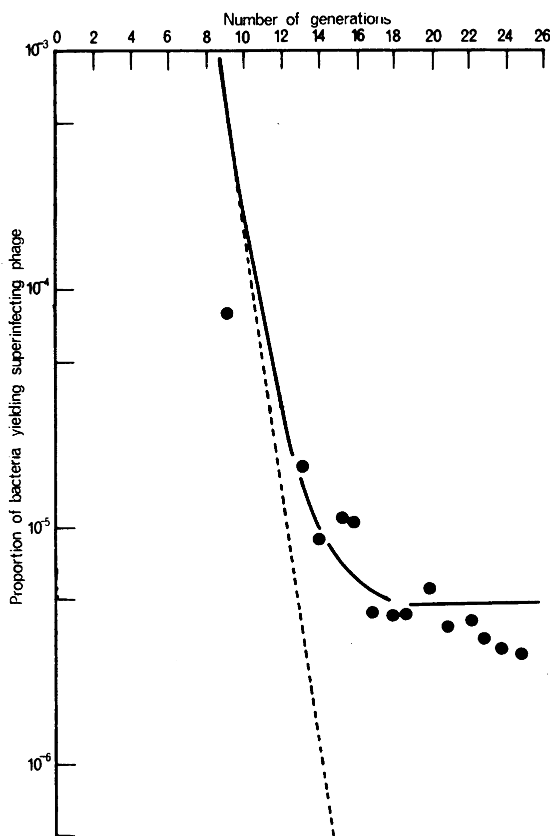


FIG. 2b.

MATERIALS AND METHODS

Phages.—The superinfecting phage was derived from a stock of phage P22 provided by Dr. T. F. Anderson. This carried the mutant markers c_2, virulent (Levine, 1957); h, ability to plate on semi-rough strains of *Salm. typhimurium* (Yamamoto and Anderson, 1961; Wilkinson, 1966); and m_3, a plaque-type marker not relevant here. A magnesium-resistant (mg^r) mutant was derived by plating on nutrient agar containing 0·1 M $MgCl_2$ (Zinder, personal communication). The lysogenizing phage also carried the mutant markers, $a1$, inability to cause synthesis of somatic antigen 1 (Young, Fukasawa and Hartman, 1964) and x, inability to exclude superinfecting phage P22 (Walsh and Meynell, 1967*a*). The genotypes of the superinfecting and lysogenizing phages were therefore $c_2h\ mg^r\ al^+\ x^+$ and $c^+h^+mg^sa1\ x$.

Bacteria.—The strain used to infect mice was SR120, a derivative of *Salm. typhimurium* strain LT2 (Zinder, 1958). This was lysogenized, and the colicin factor, ColIb-P9, also introduced (to give strain M526) in order to increase its resistance to U.V. light (Walsh and Meynell, 1967*b*) and so to lessen the risk of excessive exposure. A mutant, SL1207, of strain

SR120 resistant to 1000 μg. streptomycin/ml. was also used: its lysogenic Col+ derivative is strain M462. The bacterial indicator strains were SL1207 for the h^+ lysogenizing phage and SL1208, a *str-r* mutant of the semi-rough strain *rou*-536, for the *h* superinfecting phage.

Culture media.—Indicator cultures were grown overnight in unshaken Oxoid Nutrient Broth No. 2. Growth experiments were performed in a amixture of 3 vol. Oxoid Broth, 1 vol.

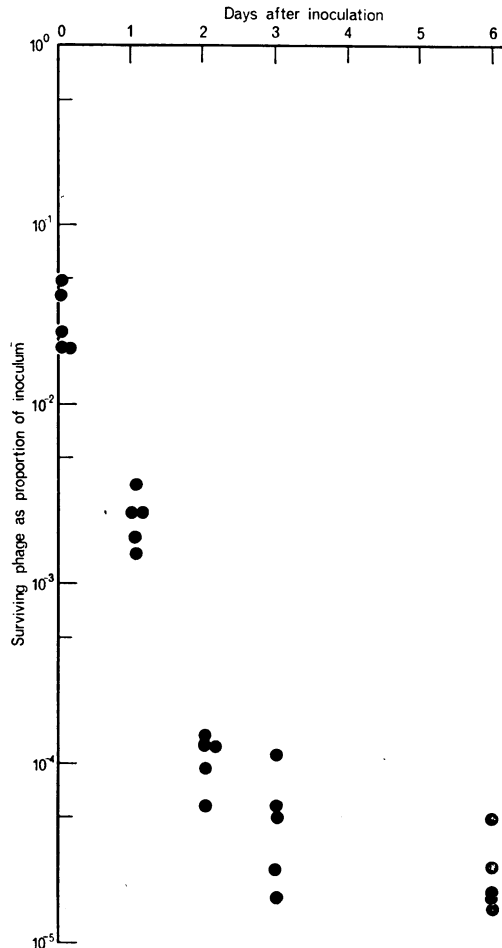


FIG. 3.—Proportion of c_2 phage recovered from the spleen after intravenous injection of $1{\cdot}4 \times 10^7$ particles per mouse.

salts medium (Lederberg, 1950) and 0·2 per cent glucose. Plaques were counted on nutrient agar containing 10 g. Bacto Tryptone, 15 g. Bacto Noble Agar and 1 g. glucose per litre de-ionized water; the glucose was added to molten agar from a sterile 20 per cent solution just before plates were poured. Overlays contained the same agar diluted with an equal volume of de-ionized water.

Preparation of inocula.—An unshaken overnight culture of the lysogenic strain was mixed with the superinfecting phage at a multiplicity of 3–5 and left at 37° to allow the phage to absorb. With the *str-s* strain, M526, unadsorbed phage was counted after 10 min. on agar containing 200 μg. streptomycin/ml., using strain 1207 as indicator. In the case of the *str-r*,

strain, M462, the concentration of free phage was estimated from the count of full-sized c_2 plaques formed by unirradiated suspensions on SL1208. In the experiments of Fig. 4, 5 and 6, the majority of the unadsorbed phage was then removed (a) by washing the super-infected cells with 100 ml. broth on a membrane filter (a.p.d. 0·8 μ) and (b) by resuspending the cells in 0·1 M phosphate buffer, pH 7·2, to which was added anti-P22 serum which was allowed to act for 10 min. at 37°. This procedure lowered the concentration of active free phage by a factor of 10^{-4}. In the remaining experiments only antiserum was used, which lowered the concentration by 10^{-2}. Finally, the cells were diluted 1/8 in buffer, and 0·2 ml. of the diluted suspension inoculated into the tail vein of each mouse. The initial proportion

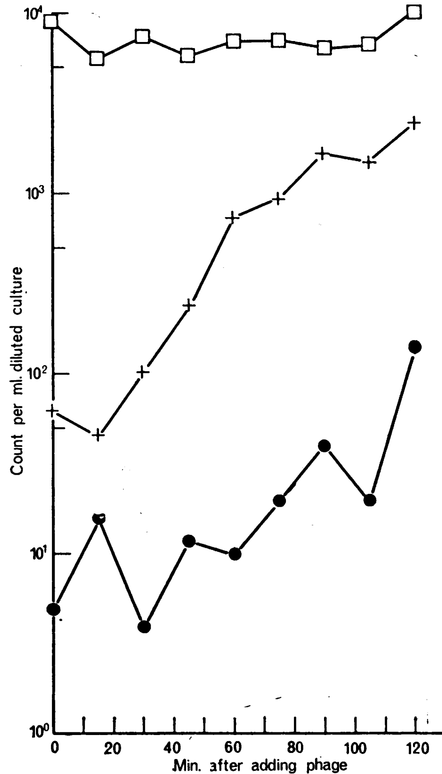


FIG. 4.—Spontaneous release of superinfecting c_2 (+) and c^+ (●) phage on incubating a super-infected culture in broth after filtration and exposure to anti-phage serum. Colony count (□). Multiplicity of infection = 12.

of superinfected cells was also measured at the same time by exposure to U.V. radiation, followed by plating in the usual way.

Estimation of the proportion of superinfected bacteria.—The bacteria, whether from cultures or from mice, were exposed to U.V. light to induce vegetative growth and plated before lysis began (1) on strain SL1207 on tryptone agar to measure the total number of cells induced, and (2) on strain SL1208 on tryptone agar containing 0·1 M $MgCl_2$ to measure the number of cells releasing the superinfecting phage. The U.V. source was a 15 w germicidal tube placed 60 cm. above a platform on which the samples were rocked during irradiation. The main technical consideration was to ensure that the effective dose of U.V. light was not diminished by U.V.-adsorbing material like broth or serum. Samples from broth cultures were therefore diluted at least 1/100 in Ringer's solution before exposure: the optimal exposure was 45 sec. Two methods were used for organisms recovered from the mouse spleen. Each spleen was

placed in 5 ml. quarter-strength Ringer's solution in a precision bore glass tube and disintegrated with a Teflon piston (Jencons, Hemel Hampstead, Herts.) driven at approximately 2000 r.p.m. by a 0·125 h.p. electric motor. In preliminary experiments (Fig. 7), U.V.-adsorbing material was removed by twice washing with Ringer's solution by centrifugation, followed by a U.V. dose of 90 sec. Later, however, the rate of U.V. inactivation of phage

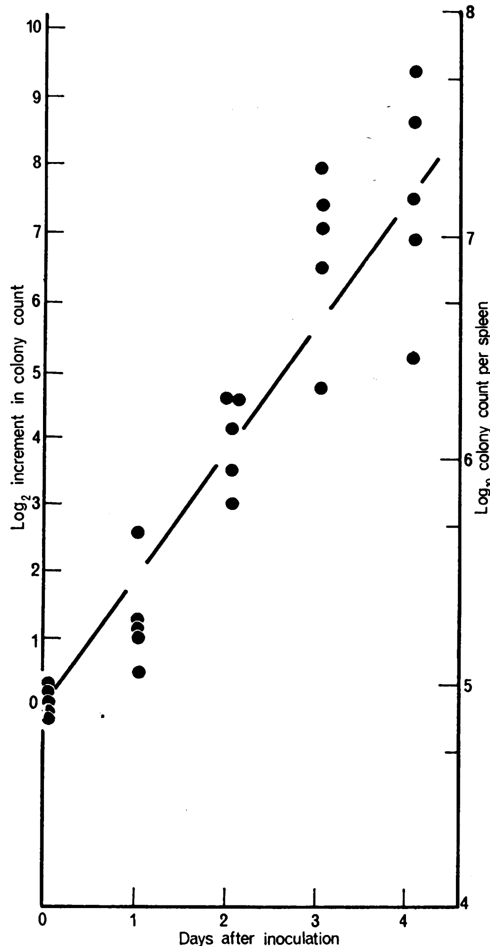


FIG. 5 (a)

FIG. 5.—Growth of the streptomycin-sensitive strain of *Salmonella typhimurium* in the mouse spleen. The dose was $1 \cdot 1 \times 10^7$ organisms per mouse, of which 1 per cent was recovered from the spleen at 1·5 hr. (Fig. 5a). Note that the number of generations tends to constancy in Fig. 5b (overleaf) due to an artefact (see Fig. 2b).

P22 suspended in spleen debris was measured and showed that adequate U.V. dosage was obtained without washing if the exposure was increased to 120 sec. In all experiments, samples were plated in overlays immediately after irradiation, the whole process being carried out in subdued daylight to avoid photoreactivation.

Calculation of the standard curve.—The shape of this curve is determined by the initial mean number of superinfecting phage genomes per bacterium which in an ideal system is equal to the mean number of superinfecting phage adsorbed per bacterium. In practice,

only a proportion of the adsorbed phage was recoverable (1–70 per cent in these experiments) and the curve had therefore to be determined in other ways. When the initial proportion of superinfected bacteria is small (< 0·1), it may be assumed to halve in each succeeding bacterial generation since the great majority of the cells each carry only one superinfecting genome initially. At somewhat higher proportions (0·1 — 0·7), a curve resembling that of Fig. 2 can be constructed from the initial proportion of cells yielding the superinfecting genome upon U.V. induction, either by an approximation, using a multi-hit curve (see curve B, Fig. 3, Meynell, 1959) or precisely, using a chart prepared for this purpose (T. Williams, personal communication). In either case, it is essential to derive the estimate of the initial proportion from counts of as many plaques as possible (*e.g.* 1000 on each indicator) because it carries a substantial sampling error, whose magnitude can be estimated graphically

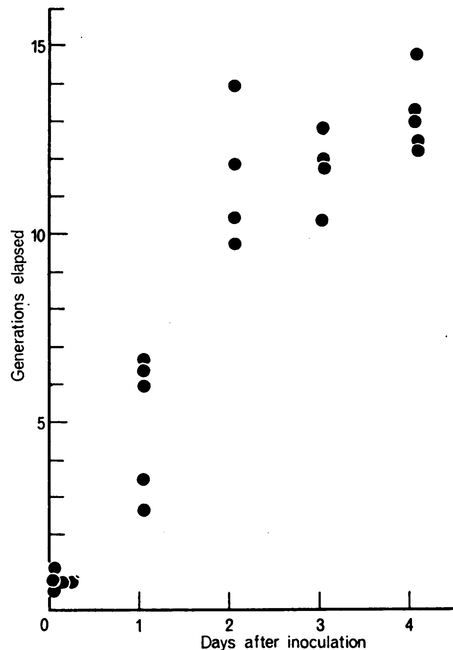


Fig. 5(b).

(Williams, 1968). Should the initial proportion exceed 0·7, it can no longer be used alone, because it is then a highly imprecise index of the mean number of superinfecting genomes per bacterium. Thus, if the mean was 3, the zero term of the Poisson series shows the proportion to be 0·95 so that however much the mean is increased, the proportion can increase only by 0·05 to 1·00. When the proportion is > 0·7 the curve should therefore be determined directly by repeated counts on part of the inoculum growing in broth, as in Fig. 2a.

RESULTS

The phage-bacterium system

As a superinfected culture passes through successive generations, relatively fewer and fewer cells contain the superinfecting phage though all carry the prophage (Fig. 1, 2). The minority of superinfecting phage will therefore be measurable only if it carries selective markers that allow it to be counted separately from the prophage. A host range (h) mutant of phage P22 is available which was originally isolated on derivatives of *Salm. typhimurium*, strain LT2, selected for

resistance to virulent mutants of phage P22 h^+ (Yamamoto and Anderson, 1961). These derivatives are now known to be semi-rough mutants (Wilkinson, 1966), and phage P22*h* formed readily countable plaques on the bacterial mutant, rou-536, chosen as indicator. However, *rou*-536 nevertheless permitted wild type ($h+$) P22 to form dim plaques, and magnesium-resistance (mg^r) was therefore used as an additional selective marker for the superinfecting phage. As the superinfecting phage was virulent (c_2) and excluding (x^+), its complete genotype was $c_2\ h\ mg^r\ x^+$, which allowed it to be counted on the mutant *rou*-536 growing on agar containing 0·1 M $MgCl_2$ (efficiency of plating of phage suspensions, 0·7) on which no plaques were formed by the lysogenizing phage of genotype $c^+h^+mg^sx$

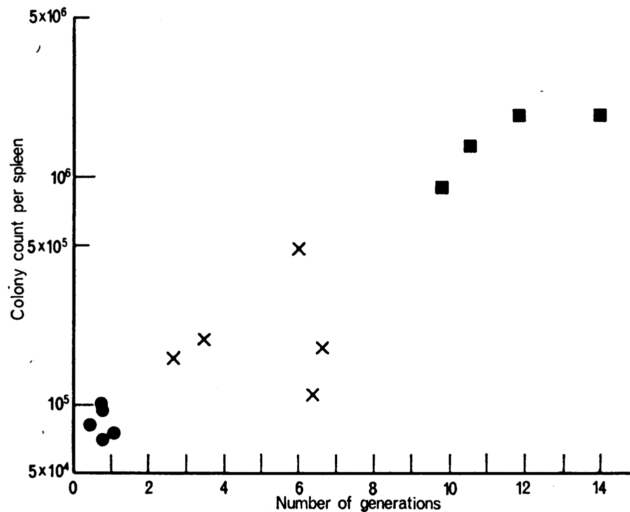


FIG. 6.—The relation between colony count per spleen and number of generations elapsed during infection by the streptomycin-sensitive strain of *Salmonella typhimurium*. Counts were made after 1·5 hr. (●), 1 day (×) and 2 days (■).

(efficiency of plating, $<10^{-8}$). The latter was counted on conventional agar using a smooth bacterial strain, SL1207, as indicator. The proportion of bacteria carrying the superinfecting phage was thus obtained from the plaque count on SL1208/the plaque count on SL1207, but could often be measured directly from the proportion of mottled plaques on strain SL1207, since mottling indicates that both c_2 and c^+ phage are released by the same cell.

In practice, only 10–30 per cent of cells are induced by irradiation but some of the non-induced cells nevertheless form small c^+ plaques, due to spontaneous induction during growth of the colony in the overlay. These are ignored in making the counts which are therefore derived solely from the numbers of full-sized c^+ plaques.

Superinfected cultures behaved as expected when grown in broth with a doubling time of 0·5 hr. (Fig. 2*a*) until the 10th generation was reached. Thereafter, the proportion of organisms yielding the superinfecting phage ceased to decrease exponentially and gradually tended to constancy (Fig. 2*b*), showing that the superinfecting genes, $h\ mg^r$, were replicating at each bacterial division. This

occurred because superinfecting phage became integrated with the chromosome of a small proportion of superinfected bacteria (*ca.* 10^{-6} in this system), possibly by recombining with the prophage to produce a doubly lysogenic organism. It is not known whether integration can only occur immediately after superinfection or whether it has a constant probability of occurrence per unit time or per bacterial generation. In any event, calculation shows that the curve will have approximately the form shown in Fig. 2*b* and that the departure from an exponential

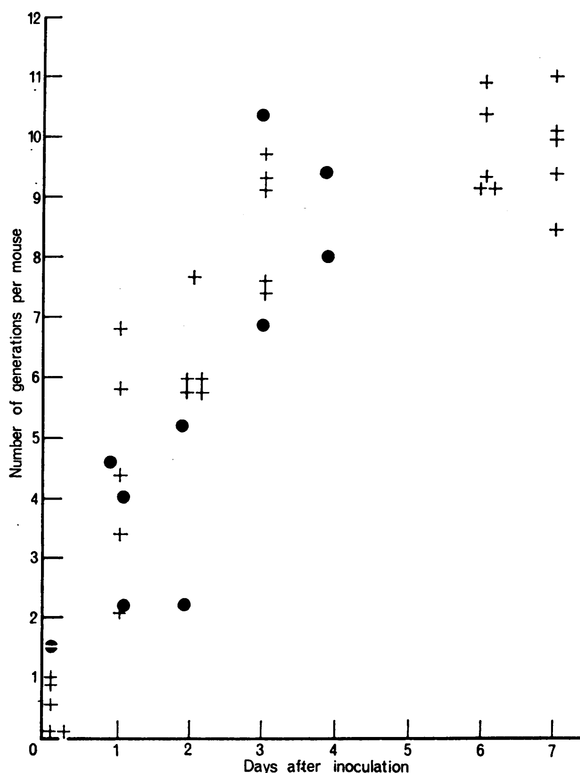


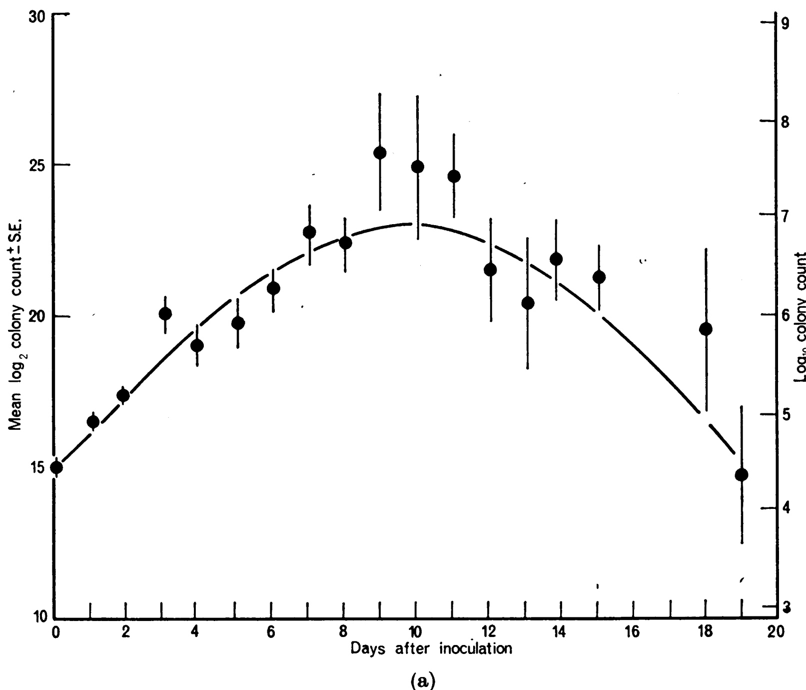
FIG. 7.—Number of generations undergone by the streptomycin-resistant strain of *Salmonella typhimurium*. In these experiments, indicated by ● and +, the spleens were washed twice with Ringer's solution before U.V. irradiation. Free c_2 phage is therefore unlikely to have disturbed the estimates of the number of generations.

decline becomes detectable once 10 per cent of cells carrying the superinfecting phage possess it in the integrated state.

The problem of free phage in vivo

As a lysogenic strain grows *in vitro*, spontaneous induction occurs in a proportion of bacteria, resulting in vegetative multiplication of both prophage and superinfecting phage and leading to their subsequent release into the medium. Spontaneous induction presumably also occurs *in vivo* and it is easy to envisage a situation in which the count of free phage arising from spontaneous induction

might exceed that derived from U.V.-induced cells, so that the plaques formed after U.V.-irradiation would not be formed solely by lysing bacteria as the method assumes. The contribution of free phage to the plaque counts was therefore estimated (1) by measuring the ability of inoculated phage to survive in the mouse spleen for, if this proved to be small, free phage would be unlikely to disturb the experiments; and (2) by counting the concentration of free phage in each homogenized spleen by plating on streptomycin agar to kill the bacteria,



(a)

FIG. 8.—Growth of the streptomycin-resistant strain of *Salmonella typhimurium* in the mouse spleen, showing the mean estimates ± S.E. mean obtained from counts on 109 mice in 4 experiments. The colony counts describe a parabola and originate from a point corresponding to 3 per cent of the dose (Fig. 8a). The mean numbers of generations given in Fig. 8b (overleaf) comprise experiments in which the spleen homogenate was washed before U.V. irradiation (●, derived from the points of Fig. 7) and those in which washing was omitted (+). The two sets of means are consistent, suggesting that negligible amounts of free c_2 phage was present.

using strain 1207 as indicator to enable superinfecting and lysogenizing phage to be distinguished by the differing morphology of their plaques.

Inoculated phage was only slowly inactivated in the spleen. About 3 per cent of the inoculum was recovered 1·5 hr. after administration and the count fell only 1000-fold in the following 3 days. Moreover, the count then became virtually constant (Fig. 3). Evidently, the host could not be relied upon to inactivate free phage as soon as it arose.

The amount of free phage in each spleen was measured in 2 experiments (Table). The lysogenizing (c^+) phage was never present to an extent that significantly disturbed the counts of U.V.-induced cells, for, even before irradiation, it contributed less than 1 per cent of the total c^+ plaques obtained after irradiation.

However, in both experiments, relatively high concentrations of free superinfecting (c_2) phage were found which, in many mice, were equal to the count of c_2 plaques obtained after irradiation. The error thus introduced is considerably less in practice than the Table suggests because 70–80 per cent of free phage is inactivated by the dose of U.V.-irradiation used for induction. In Table 1a, significant quantities of free c_2 phage occurred only occasionally but were frequent in Table 1b. The actual figures at 1·5 hr. are *ca.* 0·03 and 0·5 for Table 1a, b, respectively, compared with 0·001 for both inocula. It follows that the relative

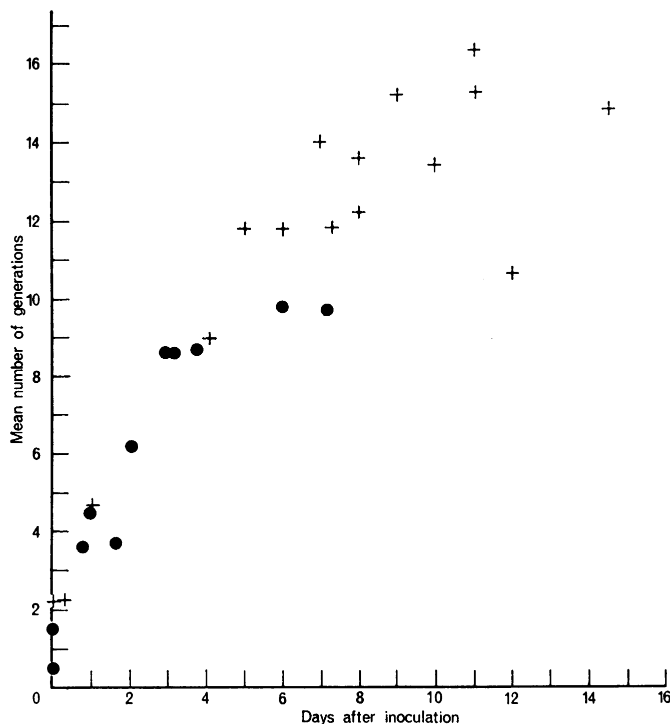


FIG. 8(b).

count of free c_2 phage increased 30–500 fold in the two experiments within 1·5 hr. of inoculation. The most obvious explanation—that inoculated free phage survived *in vivo* 30–500 times better than inoculated bacteria—is excluded, because they survived equally well, as comparison of Fig. 3, 5a and 8a will show. Furthermore, there is considerable evidence that the total free phage per mouse must have increased during the first 1·5 hr. First, it is striking in the Table that the count of free phage per spleen at 1·5 hr. was of the same order as the total number of free phage received by each mouse, although only 3 per cent of the inoculum is recovered when phage alone is injected (Fig. 3). Second, Fig. 4 shows that when superinfected cells were prepared in the usual way and then incubated in broth, the amount of free phage began to increase after 20–30 min. and that the concentration of free c_2 phage exceeded that of c^+ phage. Thus, the superinfecting phage succeeded in replicating in a minority of cells, either because

TABLE.—*Free Phage Arising* in vivo.

	1a			1b		
	Relative count* of free phage		Free c_2 phage	Relative count* of free phage		Free c_2 phage
	c^+	c_2		c^+	c_2	
	—	0·001	$1\cdot0\times10^{2}$	—	0·001	$7\cdot0\times10^{1}$
Sampled after (hr.)						
	—	0·04	$2\cdot7\times10^{2}$	—	0·33	$1\cdot3\times10^{2}$
	—	0·02	$1\cdot5\times10^{2}$	—	0·5	$1\cdot7\times10^{2}$
1·5	—	0·02	$1\cdot5\times10^{2}$	—	0·1	$9\cdot0\times10^{1}$
	—	—	—	—	0·45	$1\cdot2\times10^{2}$
	—	0·02	$1\cdot7\times10^{2}$	—	0·75	$2\cdot3\times10^{2}$
	—	0·05	$5\cdot0\times10^{1}$	—	1·5	$5\cdot0\times10^{1}$
	—	0·36	$1\cdot0\times10^{2}$	—	1·0	$8\cdot5\times10^{1}$
26	—	0·1	$1\cdot0\times10^{2}$	0·07	1·0	$9\cdot3\times10^{0}$
	—	0·03	$2\cdot5\times10^{1}$	—	0·66	$5\cdot0\times10^{1}$
	—	0·15	$5\cdot0\times10^{1}$	—	—	—
	0·005	0·88	$7\cdot5\times10^{1}$	0·07	0·9	$7\cdot8\times10^{0}$
	0·003	0·16	$2\cdot0\times10^{1}$	—	0·9	$7\cdot8\times10^{0}$
48	0·003	0·2	$2\cdot5\times10^{1}$	0·002	2·0	$1\cdot5\times10^{1}$
	0·006	0·56	$6\cdot0\times10^{1}$	—	1·0	$5\cdot0\times10^{1}$
	0·002	1·1	$2\cdot0\times10^{1}$	—	0·26	$1\cdot5\times10^{1}$
	0·006	0·44	$1\cdot4\times10^{2}$	0·004	—	—
	0·004	1·1	$3\cdot7\times10^{2}$	—	—	—
74	0·006	0·3	$9\cdot0\times10^{1}$	—	—	—
	0·003	0·2	$1\cdot3\times10^{2}$	0·002	—	—
	0·002	0·09	$3\cdot0\times10^{1}$	0·0007	—	—
	0·001	—	—	0·002	—	—
	0·0009	0·09	$6\cdot0\times10^{1}$	0·0009	—	—
98	0·009	—	—	—	—	—
	0·002	0·16	$6\cdot0\times10^{1}$	0·0006	—	—
	0·003	0·19	$1\cdot0\times10^{1}$	0·006	—	—

* The relative count of free phage = plaque count on streptomycin-agar before U.V.-irradiation/ total plaques of a given kind formed on nutrient agar after irradiation.

— = no plaques formed on streptomycin agar.

induction occurred spontaneously or because the number of superinfecting genomes entering those cells was large enough to overcome their immunity, so causing vegetative phage growth.

The unwanted free phage arising in this way might be eliminated either by incubating the superinfected cells in broth for 1–2 hr. before filtration and treatment with antiphage serum; by neutralizing the free phage in each spleen homogenate with antiserum (although this would subsequently have to be removed—*e.g.* by dilution—to prevent significant carryover into the overlays used for plaque counts); or by repeatedly washing the ground spleen suspension by centrifugation, as done here in certain experiments.

Kinetics of infection in the spleen

Two sets of experiment were done, using a streptomycin-sensitive strain, M526, and a resistant strain, M462.

Streptomycin-sensitive organisms.—The mice of Table 1a were infected by injecting $1\cdot1\times10^{7}$ organisms into a tail vein. The mean colony count per spleen 1·5 hr. later was 10^{5} or 1 per cent of the dose (Fig. 5a). No deaths occurred before the 4th day when the experiments were ended. Between 1·5 hr. and 4 days, the colony count appeared to increase exponentially, as is usually found (Meynell

and Meynell, 1958; Williams and Meynell, 1967). The growth equation is therefore

$$N_t = N_i 2^{(l-m)t}$$

where N_t is the mean colony count at time t after inoculation, N_i is the mean colony count 1·5 hr. after inoculation, and l–m is the net rate of increase of the organisms. The left-hand ordinate of Fig. 5a shows that N increased 2^7 times in 4 days so that, taking 1 days as the time unit, the value of l–m was $7/4 = 1{\cdot}75$.

The numbers of generations elapsed at various times after inoculation are shown in Fig. 5b and allow the value of l to be estimated. Three stages can be distinguished:

(1) The initial 1·5 hr. (0·062 days), in which an average of 1 generation elapsed. This corresponds to $l = 1/0{\cdot}062 = 16$. By comparison, the maximum growth rate observed *in vitro* is 48, corresponding to a doubling time of 30 min.:

(2) An intermediate stage extending from 1·5 hr. to 2 days in which an average of 10 generations elapsed, giving $l = 5$. Since l–m was 1·75, the value of m was $5 - 1{\cdot}75 = 3{\cdot}25$.

(3) The last stage from the 2nd day onwards, in which the number of generations appeared to become constant, although the colony count was still increasing at the same rate! (Fig. 5a). This result is an artefact, due to the proportion of bacteria yielding superinfecting phage becoming constant owing to the outgrowth of that small fraction of bacteria in which the superinfecting phage became integrated in the bacterial genome. This fraction became manifest *in vitro* after 10–12 generations (Fig. 2b), which corresponds to the third stage apparent in Fig. 5b.

The counts on individual mice were analysed further, to see if an above-average colony count on a given day was associated with an above-average value of l or with a below-average value of m. Fig. 6 shows that no definite association existed. The 5 mice killed at 1·5 hr. differ very little, which is unusual since gross differences in individual colony counts are generally present within a short time of inoculation (Meynell and Maw, 1968). However, the mice killed after 1 and 2 days, respectively, show that both l and m must differ from mouse to mouse. Thus, after 1 day there were 3 mice in which 6 generations had elapsed, yet their colony counts differed over a 5-fold range, showing that l was the same but m differed for this group. On the other hand, there were 3 mice with colony counts of *ca.* 2×10^5 although the number of generations elapsed varied from 2·5–6·5, indicating that, for this group, both l and m differed.

Streptomycin-resistant organisms.—In these experiments, the concentration of free phage in the spleen could not be measured by plating on streptomycin-agar. However, the estimated numbers of generations is unlikely to have been distorted by free phage because the estimates were unaffected by washing the homogenized spleens twice before irradiation which in itself inactivates 70 per cent of free phage. Fig. 7 shows the estimates for individual mice in 2 such experiments. The average colony counts and estimates are given in Fig. 8a, b respectively. In Fig. 8b, the points based on spleens washed before irradiation are distinguished from those for spleens irradiated immediately: the two sets are clearly consistent. It will be noted that these experiments lasted for 14–19 days. Life tables showed that about 27 per cent of mice died in the 12 days following inoculation, usually on the 7, 8 or 9th day.

As with the streptomycin-sensitive strain (Fig. 5), 3 phases can be distinguished, although division occurred more slowly and the course of infection was more protracted. In the first 1·5 hr., about 1·5 generations elapsed, giving $l = 1{\cdot}5/0{\cdot}062 = 24$. A second phase lasting from 1·5 hr. to about 5 days in which 10 generations occurred, giving $l = 10/4$ days $= 2{\cdot}5$. And the final phase in which the true division rate appeared to fall, presumably because of the outgrowth of organisms carrying the superinfecting genome in the integrated state.

The colony counts increased 2^5 times in the 5 days of the second phase (Fig. 8a), giving a net rate of increase $(l–m) = 5/5 = 1$. The corresponding value of m, the true death rate of organisms in the spleen, is $l - (l–m) = 2{\cdot}5 - 1 = 1{\cdot}5$.

DISCUSSION

The findings of these experiments relate, first, to the technique and, second, to the behaviour of the organisms *in vivo.*

The superinfecting phage technique

It is evident that this can now be applied to a variety of salmonella infections. Phage P22 adsorbs to many salmonella species other than *Salm. typhimurium*, provided they possess somatic antigen 12 (Lederberg and Edwards, 1953), any of which can be used in this type of experiment now that non-excluding phage mutants are available (Walsh and Meynell, 1967*a*) with efficient selective markers for the superinfecting phage. The major technical simplification introduced since the original method (Meynell, 1957) is the induction of phage growth by irradiating unwashed spleen suspensions, which avoids the considerable labour of repeated centrifugation, although this may continue to be needed to remove free phage accumulating *in vivo.*

There are two features of the present phage-bacterium system which might be improved by introducing further mutations into the phages. One concerns the integration of superinfecting phage which, although occurring in only a minute proportion (10^{-6}) of the bacteria (Fig. 2b), progressively disturbs the method after some 10 generations have elapsed. The ability of phage P22 to integrate is largely absent in L mutants (Smith and Levine, 1967), and we therefore tested the 4 possible combinations of L^+ and L superinfecting phage and prophage to see if the L mutation diminished integration after superinfection. Integration appeared to occur somewhat less frequently when both phages carried the mutant L gene but the results were not sufficiently promising to be worth pursuing. It will nevertheless be appreciated that 10 generations represents a 1000-fold dilution of the marker particles in the infecting bacterial population so that, even within the limits imposed by integration, the present method is highly sensitive. The reason is that it depends on a biological marker—the superinfecting phage genome —which, unlike a physical marker such as an isotope, can be induced to replicate at will which enables even a single marker particle to be readily detected, in this case, as a plaque.

The second feature of the present phage-bacterium system which it would be desirable to eliminate is the proneness of the superinfecting phage to undergo spontaneous vegetative growth in a minority of cells. This may be inherent in the nature of the immunity conferred by a prophage to superinfection. Immunity

is mediated by molecules of repressor (Ptashne, 1967) whose effect can be overcome by increasing the relative number of superinfecting genomes per cell (Jacob, Sussman and Monod, 1962; Weismeyer, 1966). The mouse experiments require a moderately high multiplicity of infection in order to be able to measure a reasonable number of generations *in vivo*, but it follows from the random adsorption of superinfecting phage to the bacteria that a few cells will receive substantially above-average numbers of particles. Thus, if the m.o.i. is 2, 0·005 per cent of cells each adsorb 10 or more phage; at a m.o.i. of 4, the figure rises to 1 per cent. Considering the burst size after infection of non-lysogenic cells may be 500–1000, it seems likely that the c_2 phage spontaneously liberated after superinfection arises from the minority of cells that chanced to adsorb large numbers of phage.

The behaviour of the organisms in the spleen

The most striking finding is that l and m, the true rates of bacterial division and death, are each small, so that the whole course of the infection appears relatively sluggish. The mean value of l of 2·5–5 shows the organisms to be dividing at only 5–10 per cent of the maximum rate found in broth, corresponding to doubling times of 10–5 hr. compared to 30 min. That the value of m should be only 1·5–3·25 is even more conspicuous, for bactericidal processes studied *in vitro* operate at rates which are several orders of magnitude greater. Thus, if 50 per cent of a non-dividing culture died in 30 sec., the value of m would be 2880. The resistance of bacteria to killing by many agents is partly determined by their division rate (see Meynell, 1961). The slow division of the organisms in the spleen may therefore be directly responsible for their small death rate: *i.e.* if the organisms had divided faster, they might have been killed faster. This can be viewed in another way. The infections prevalent today presumably represent a balance between host and pathogen in which neither is invariably eliminated. It is conceivable that this balance is reflected here by host processes which favour the host by restraining bacterial division and, at the same time, enable the pathogen to survive bactericidal processes that would eliminate more rapidly-dividing cells.

A major obstacle to the experimental analysis of infection is that the organisms are growing in an almost completely uncharacterized environment which, by its nature, is virtually impossible to observe directly. One solution to this problem is to study the kinetics of infection (Meynell and Stocker, 1957; Meynell and Meynell, 1958; Meynell, 1963; Armitage, Meynell and Williams, 1965; Williams and Meynell, 1967) where one allows the infection to proceed undisturbed with the aim of asking how the organisms behave, as distinct from passing immediately to the more usual but necessarily far more complex question of why they behave as they do. The superinfecting phage technique is therefore an adjunct to kinetic studies, for its primary purpose is to show how fast infecting bacteria divide and are killed within their host, without enquiring into the mechanisms responsible. It is clear, however, that once the kinetics have been established, one should be in a better position to identify the factors controlling microbial growth *in vivo* and that the two approaches are complementary.

We should like to thank Dr. N. D. Zinder for kindly providing us with details of his unpublished work with magnesium-resistant mutants of phage P22.

SUMMARY

The rate at which *Salm. typhimurium* divides and is killed *in vivo* can now be determined, following the isolation of mutants of phage P22 for use in the super-infecting phage technique. The division rate of the organisms in the spleen of mice challenged by intravenous injection proved to be only 5–10 per cent of the maximum observed *in vitro*. Their death rate in the spleen was also extremely small. The technique is applicable to other Salmonella species.

REFERENCES

ARMITAGE, P., MEYNELL, G. G. AND WILLIAMS, T.—(1965) *Nature, Lond.*, **207**, 570.
BERTANI, G.—(1954) *J. Bact.*, **67**, 696.
JACOB, F.—(1954) *Les Bactéries Lysogènes et la Notion de Provirus.* Paris (Masson).
JACOB, F., SUSSMAN, R. AND MONOD, J.—(1962) *C.r. hebd. Séanc. Acad. Sci., Paris,* **254**, 4214.
LEDERBERG, J.—(1950) *Meth. med. Res.*, **3**, 5.
LEDERBERG, J. AND EDWARDS, P. R.—(1953) *J. Immun.*, **71**, 232.
LEVINE, M.—(1957) *Virology*, **3**, 22.
MEYNELL, G. G.—(1959) *J. gen. Microbiol.*, **21**, 421.—(1961) *Symp. Soc. gen. Microbiol.*, **11**, 174.—(1963) *Nature, Lond.*, **198**, 960.
MEYNELL, G. G. AND MAW, J.—(1968) *J. Hyg. Camb.*, **66**, 273.
MEYNELL, G. G. AND MEYNELL, E. W.—(1958) *J. Hyg. Camb.*, **56**, 323.
MEYNELL, G. G. AND STOCKER, B. A. D.—(1957) *J. gen. Microbiol.*, **16**, 38.
MEYNELL, G. G. AND SUBBAIAH, T. V.—(1963) *Br. J. exp. Path.*, **44**, 197.
PTASHNE, M.—(1967) *Nature, Lond.*, **214**, 232.
REES, R. J. W. AND HART, P. D.—(1961) *Br. J. exp. Path.*, **42**, 83.
SMITH, H. O. AND LEVINE, M.—(1967) *Virology*, **31**, 207.
WALSH, J. AND MEYNELL, G. G.—(1967*a*) *J. gen. Virol.*, **1**, 581.—(1967*b*) *J. gen. Virol.*, **1**, 587.
WIESMEYER, H.—(1966) *J. Bact.*, **91**, 89.
WILKINSON, R. G.—(1966) Rough mutants of *Salmonella typhimurium*. Ph.D. thesis. University of London.
WILLIAMS, T.—(1968) *J. gen. Virol.*, **2**, 13.
WILLIAMS, T. AND MEYNELL, G. G.—(1967) *Nature, Lond.*, **214**, 473.
YAMAMOTO, N. AND ANDERSON, T. F.—(1961) *Virology*, **14**, 430.
YOUNG, B. G., FUKAZAWA, Y. AND HARTMAN, P. E.—(1964) *Virology*, **23**, 279.
ZINDER, N. D.—(1958) *Virology*, **5**, 291.