REACTIVATION OF IRRADIATED BACTERIOPHAGE BY TRANSFER OF SELF-REPRODUCING UNITS

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In investigating certain peculiarities in the titration of the activity of bacteriophages after ultraviolet irradiation, a mechanism was discovered that offers direct support for interpreting inactivation of these viruses by radiation as due to lethal mutations. Moreover, this mechanism reveals unexpected features of the process of virus reproduction. We summarize here the results of this work and discuss some of their implications. They will be published in detail elsewhere.

Our work employed the coli bacteriophages, T1–T7, their "r" mutants (i.e., with rapid lysis, and large plaque), the host strain, Escherichia coli B, and a number of bacterial mutants resistant to one or another of the bacteriophages.[1, 2]

The rate of inactivation of these bacteriophages exposed to ultraviolet light (wave-length 2537 A) in a non-absorbent medium is a simple logarithmic function of the dose of irradiation (Fig. 1).[3,4] This indicates a "one hit" mechanism of inactivation, one quantum being the effective inactivating hit. The phage survival is generally measured by plaque count, by diluting the irradiated phage rather heavily in broth and plating the diluted samples with sensitive bacteria. Each phage particle that remains active will give one plaque (phage colony).

It was, however, noticed by Delbrück and Bailey (personal communication) that the active titre of an irradiated suspension, as determined by plaque count, is dependent on the concentration of the irradiated sample when first placed in contact with the bacterial cells. For example, an irradiated sample can be diluted 1:10 into a heavy bacterial suspension, and after a few minutes—during which no phage liberation occurs—diluted again $1:10^4$ in broth and plated. The count will be much higher than if the same sample had first been diluted $1:10^4$ in broth, then 1:10 in bacterial suspension. This phenomenon seemed to indicate the presence in irradi-

ated phage suspensions of some "factor," besides the phage itself, capable of causing reactivation of inactivated phage particles if allowed to act on the same bacterial cells. A study of this factor was the original purpose of the work reported here.

It has been shown previously,[5] and again in the experiments discussed in this paper, that ultra-violet inactivated phage particles are still adsorbed by sensitive bacteria at the same rate as active particles, even after having received as many as 40–50 lethal hits. This can be demonstrated because
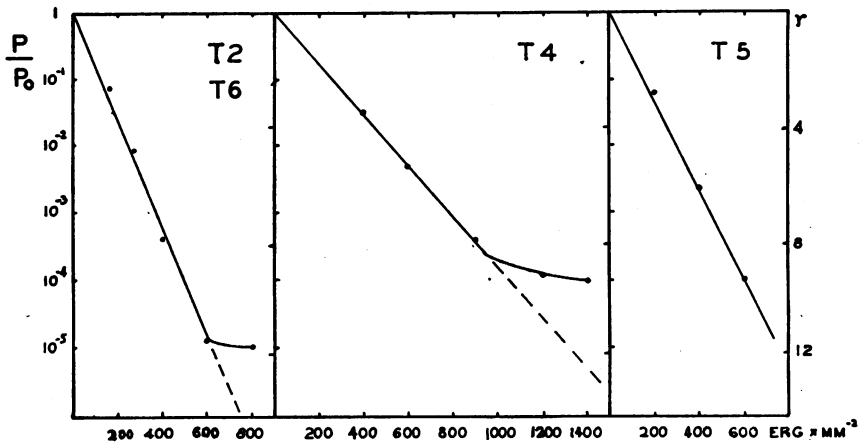

FIGURE 1

Survival curves for phages T2, T6, T4, T5 in synthetic medium. P/P_0 = proportion of active phage particles after irradiation. $r = \ln P_0/P$ = average number of lethal hits per particle. The deviations for high doses are due to the reactivation (described in this paper) taking place on the assay plates.

each bacterial cell that adsorbs one phage particle fails to divide and to produce a colony after plating. Incidentally, this fact makes it possible to calculate the number and rate of adsorption of ultraviolet inactivated phage particles. The unknown reactivating "factor" must be something that, when acting on a bacterial cell that has adsorbed an inactive phage particle, causes the production of active phage.

We first established that reactivation only occurs for the large-particle phages T2, T4, T6, T5, and their mutants, not the small phages T1 and T7. It was found next that the "factor" is partially phage specific, in the sense that even a concentrated suspension of one phage can generally not reactivate the particles of another irradiated phage. This can be tested by preparing mixtures of host cells with two phages in various proportions, the irradiated phage whose reactivation is to be tested being so diluted that

little or no reactivation would take place in the absence of the other phage suspension. After a few minutes of contact, the mixtures are diluted and plated with bacteria sensitive only to the phage whose reactivation is being tested. Since all phage suspensions are lysates of the same host cells, the specificity of reactivation suggests that the "factor" is not of bacterial origin.

An important exception to specificity is that cross-reactivation occurs between suspensions of phages of the T-even group (T2, T4, T6). These phages, although representing different wild types and distinguishable by a number of characteristics, are known to be serologically related and morphologically similar. Moreover, particles of these phages are known to be capable of mutual transfer of hereditary characteristics when adsorbed by the same host cell.[6] Reactivation of one of these T-even phages can be induced both by irradiated and non-irradiated suspensions of any of the other T-even phages.

These results suggested that the reactivating "factor" might simply be phage itself, in the sense that an inactive phage particle, if adsorbed on the same cell with another particle of the same or of a related strain, could be reactivated by transfer from the latter of the genetic locus or loci at which lethal mutations had occurred.

This hypothesis was qualitatively supported by the finding that cross-reactivation between two related phages can only occur in the presence of bacteria capable of adsorbing both phages, not in the presence of bacteria sensitive only to one of them. For example, T6 can reactivate T2 in presence of strain B, sensitive to both, but not in presence of bacteria B/6, sensitive to T2 and resistant to T6.

That reactivation is not caused by some other "factor" in the lysates was also supported by the finding that reactivation occurred with phage that had been purified from extraneous material by differential centrifugation (Strain T4r, kindly supplied by Dr. T. F. Anderson).

For quantitative testing, our hypothesis can be formulated more exactly by stating that reactivation should only occur inside bacterial cells that adsorb two or more bacteriophage particles (multiple-infected bacteria). This expectation can be tested for each phage by using several mixtures of bacteria and irradiated phage in different proportions, and calculating for each mixture the number of bacteria that adsorb more than one phage particle. If the average number of phage particles adsorbed per cell is x, the proportion of cells with more than one phage particle is given by the expression: $1 - (x + 1)e^{-x}$. For x small, this expression corresponds very closely to the proportion of cells with two phage particles. The values for multiple-infected bacteria thus obtained can then be compared with the actual numbers of bacteria that liberate phage in each mixture. These numbers are measured by plating a sample for phage plaque count before lysis

TABLE 1

RELATION OF THE NUMBER OF BACTERIA YIELDING PHAGE TO THE NUMBER OF MULTIPLE-INFECTED BACTERIA

Series of mixtures containing the same number of bacteria and different numbers of phage particles were kept 10 minutes at 37°C., then plated for plaque count. The number of multiple-infected bacteria in each mixture was calculated from the formula

$$[B > 1P] = [1 - (x + 1)e^{-x}][B],$$

where $[B]$ = total number of bacteria per ml.; $[B > 1P]$ = multiple-infected bacteria per ml.; x = ratio "adsorbed phage/bacteria" in each mixture.

Expt. No.	41				43				38			
Phage	T4				T4				T6			
Dose. erg × mm.$^{-2}$	600				900				500			
x	$[B > 1P]$ (Calc.)	Count (Exper.)		Ratio 1/y (Calc.)/(Exper.)	$[B > 1P]$ (Calc.)	Count (Exper.)		Ratio 1/y (Calc.)/(Exper.)	$[B > 1P]$ (Calc.)	Count (Exper.)		Ratio 1/y (Calc.)/(Exper.)
0.67	1.7×10^8	9×10^7		1.9								
0.33	5.0×10^7	3×10^7		1.8								
0.135	1.1×10^7	6×10^6		1.9								
0.067	2.6×10^6	1.3×10^6		2.0								
0.033	6.8×10^5	3×10^5		2.1								
0.013	1.7×10^5	1×10^5		1.7								
0.95					1.9×10^8	1.7×10^7		11				
0.48					6.5×10^7	5.4×10^6		12				
0.24					1.9×10^7	1.5×10^6		13				
0.09					3.2×10^6	2.2×10^5		14				
0.05					9.0×10^5	8.0×10^4		11				
0.62									1.6×10^8	3.9×10^7		4.1
0.31									3.6×10^7	1.2×10^7		3.0
0.155									1.3×10^7	3.2×10^6		4.1
0.062									2.4×10^6	6.7×10^5		3.6
0.031									5.4×10^5	1.3×10^5		4.2
0.015									1.7×10^5	4.1×10^4		4.1
Av.				1.92				12.2				3.85
σ				0.16				1.2				0.46

occurs; every bacterium that liberates active phage will produce one plaque.

If the hypothesis is correct, the plaque counts should be directly proportional to the numbers of multiple-infected bacteria, the factor of proportionality depending on the dose of irradiation in a way that will be discussed later. A large number of experiments of this type gave results agreeing remarkably well with the expectation. Representative results are given in table 1. It was also found in these experiments that bacteria infected with more than one inactive phage particle liberate a roughly normal yield of fully active particles.

Our hypothesis can now be elaborated to give expectations for the actual values of the factor of proportionality between the numbers

of multiple-infected bacteria and of bacteria that yield active phage.   We shall assume that a phage particle contains a certain number of different self-reproducing "units" (loci), each capable of undergoing a lethal mutation under the action of radiation.   Reactivation depends on transfer of these units, the requirement for reactivation being that a given locus does *not* carry a lethal mutation in *all* of the particles that infect the same cell. The probability that this requirement is satisfied will depend on the dose of radiation, since the probability that a lethal hit takes place at the same locus in all infecting particles will increase with increasing doses of radiation. This hypothesis is graphically described in figure 2.   It can be formulated mathematically by assuming that there are in each particle of a given phage n units each capable of giving a lethal mutation.   We further assume, in first approximation, that the sensitivity of all units to radiation is the same.
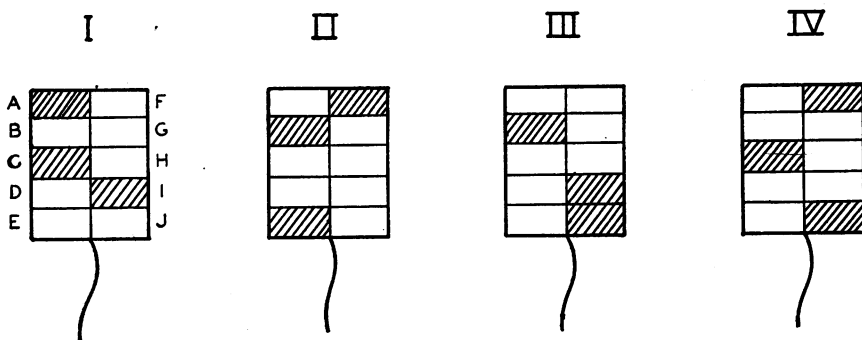

FIGURE 2

Schematic representation of phage particles, each carrying three lethal mutations, distributed at random among ten loci.

A bacterium infected with particles (I + II) will yield active phage.
A bacterium infected with particles (I + III) will not yield active phage.
A bacterium infected with particles (I + IV) will not yield active phage.
A bacterium infected with particles (III + IV) will not yield active phage.
A bacterium infected with particles (I + III + IV) will yield active phage.

The probability y_k that none of the n units carries a lethal mutation in all of k particles adsorbed by the same cell is then found[7] to be

$$y_k = [1 - (1 - e^{-r/n})^k]^n \qquad (1)$$

where r is the average number of lethal hits or mutations per particle.   The value of r can be read directly from the regular survival curve, as given in figure 1, r being the natural logarithm of the ratio between the initial titre and the survival: $r = \ln P_0/P$.

For $r/n \ll 1$—that is, for low doses of radiation—the formula (1) can be simplified with good approximation to

$$y_k \approx e^{-r^k/n^{k-1}} \qquad (1') \quad \cdot$$

In particular, for bacteria that adsorb only two phage particles ($k = 2$), the probability becomes

$$y_2 = [1 - (1 - e^{-r/n})^2]^n \qquad (2)$$

$$y_2 \approx e^{-r^2/n} \qquad (2')$$

It must be noticed that for low doses, when the probability that a lethal mutation at any given locus occurs in all particles is small, the values of y should tend to one if the efficiency of the transfer mechanism is complete, that is, if there is full recombination of active units to form active phage particles.

The formulas (1, 1') and (2, 2') can be tested experimentally by studying the dependence of the probability of reactivation (see table 1, columns $1/y$) on the dose of radiation. In particular, y_2 can be obtained from experiments with bacteria in such excess that the probability of infection higher than double can be neglected. From the values of r and y, n can be determined. If our hypothesis is correct, y should tend to one for low doses, and the values of n obtained from all experiments should be constant for each phage. A large number of experiments gave results agreeing with these

TABLE 2

CALCULATION OF THE NUMBER OF "UNITS" PER PHAGE PARTICLE (EXPERIMENTS WITH LOW DOSES)

r = lethal hits per particle; y = proportion of multiple-infected bacteria that yield phage; n = number of units per particle calculated from the formula

$$y = [1 - (1 - e^{-r/n})^2]^n \approx e^{-r^2/n} \text{ (for } r/n \ll 1). \quad n = r^2/\ln(1/y).$$

PHAGE	r	$1/y$	n	PHAGE	r	$1/y$	n
T2–T2r	2.6	1	..	T6	3.6	1	..
	4.8	1.6	49		7.9	3.4	51
	7.7	3.5	47		9.4	5.7	50
	11.0	19	41		11.7	17	49
	14.3	93	45		12.6	40	43
		Av.	45.5		15.5	70	57
						Av.	50
T4–T4r	2.8	1	..	T5	2.8	1.9	12
	5.1	2.8	26		6.1	16	13
	7.8	5.7	36		9.2	67	19
	10.0	21	33			Av.	15*
		Av.	32				

* Values uncertain, due to very low adsorption rate of this phage.

expectations. Some experiments are shown in table 2, where $1/y$ is given instead of y for convenience. We may then conclude that active phage is

formed from inactive particles by a highly efficient mechanism of transfer of any one of a relatively large number of independently transferable units: 45–50 for phages T2 and T6, 30–35 for phage T4, possibly around 15 for phage T5.

Formula (1) requires that the probability of reactivation increases with increasing values of k, that is, of the number of phage particles adsorbed per bacterium. This was verified by experiments in which bacteria were mixed with larger amounts of irradiated phage (x higher),[8] so that the probability of three or more phage particles being adsorbed by the same cell became appreciable. Representative data are given in table 3, and are seen to agree with the expectation that the values of y increase with increasing values of x (and of k).

TABLE 3

DEPENDENCE OF THE PROBABILITY OF REACTIVATION ON THE MULTIPLICITY OF INFECTION

DOSE, ERG × MM.$^{-2}$......................			400	600	800	1000
EXPT. NO.	PHAGE	x	$1/y$	$1/y$	$1/y$	$1/y$
71	T6	0.11	..	8.1	41	...
		0.55	..	7.8	25	130
		1.1	..	4.1	18	44
		2.7	..	5.1	15	44
77	T2	0.39	2.6	10.6	60	...
		0.78	2.4	9.3	35	...
		3.9	2.0	3.4	10	...
		7.8	1.65	2.15	5.3	...

These data permitted us to obtain a rough independent estimate of n from the probability of reactivation in bacteria with more than two phages. The values thus obtained for n are in sufficient agreement with those given in table 2, although generally a little lower. This systematic deviation seems to indicate that the efficiency of transfer in the case of infection with more than two particles is lower than 100%.

Our next question is the following: Does all inactivation of phage particles by ultraviolet light occur through production of lethal mutations in transferable units, or will some other mechanism of inactivation appear for very high doses? This point was investigated by using heavy doses (up to 40–50 lethal hits per particle) and comparing the results with the expectations from formula (2) for double infection. Any additional mechanism of inactivation appearing at high doses should result in a systematic deviation in the sense of less reactivation than predicted by the formula. No such deviation was found; this indicated that, even for large doses of radiation, all effect can be accounted for by lethal mutations in the transferable units.

This conclusion also has another interesting feature: it indicates that there is no appreciable amount of "linkage" in the transfer process. Any tendency of units to be transferred in groups rather than individually should give a deviation in the direction of too low reactivation at high doses, when the probability of producing two or more lethals within a linkage group becomes appreciable.

The next step was an attempt to elucidate the mechanism of transfer. The obvious analogy with crossing-over might suggest that the two entering particles exchange units directly, before any multiplication takes place, and that what then multiplies is a fully active particle resulting by. exchange. This possibility is difficult to reconcile with the very high efficiency of reactivation: in case of crossing-over we would hardly expect that all active units be collected into one active particle. Another test can be made by the cross described in figure 3. Remembering that an active particle of one phage may reactivate an inactive particle of another phage, we may expect that transfer will also occur between active and inactive particles of the same phage. If there is direct crossing-over, an active particle, if adsorbed with inactive ones onto the same cell, should often be inactivated by transfer of some "lethal" unit.
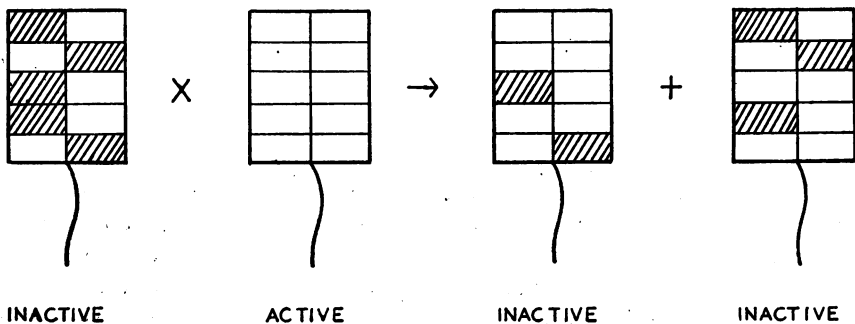

FIGURE 3

Schematic representation of the expectation in case of "cross" between a heavily irradiated particle and an active particle, assuming transfer of units by direct crossing-over between the two infecting particles.

In the actual experiments, phage suspensions were given large doses of radiation (20–50 hits per particle). Mixtures were set up containing bacteria, inactive phage, and active phage in proportion such that a large number of cells received one active and one or more inactive particles. If there were direct exchanges before reproduction, the result should be the one described in figure 3. A high proportion of the infected cells should end up with two inactive particles and, therefore, should yield no active

phage. This was not found to occur in any case: an active phage particle
was never inactivated by "crossing" with an inactive one.[9]

A possible alternative interpretation of the transfer is that each of the active units can reproduce copies of itself in excess of the number needed for multiplication of the phage particle as a whole. The copies of each unit might then either become incorporated into other phage particles that missed them, or come together to reconstitute active phage particles, independently of the origin of the individual units from one or another of the infecting particles. We have no evidence as yet available whether the "lethal" units do not multiply, or multiply in a way that makes them unfit for their niche in the phage particle. In view of the high efficiency of the reactivation process and of the approximately normal yield of phage particles, it seems certain that, if the inactive units reproduce at all, they do not have any appreciable chance of becoming incorporated into the newly formed particles.

We also have no evidence as yet as to whether the active units in an inactive particle will proceed to multiply in single-infected cells. The same may be said for the question whether the active units remain in spatial relation to each other, as it were, in a "frame phage particle," while reproducing excess copies that are incorporated into the new particles that are formed. Work to attack some of these problems is now in progress.

A point of considerable interest is that in numerous experiments in which phage particles were inactivated by x-rays instead of ultraviolet light, no reactivation occurred, although the x-ray inactivated particles were still adsorbed by the bacterial cells.

Discussion: These experiments have suggested a possible mechanism of reproduction of phage particles inside the host cell, according to which reproduction would take place, as it were, in an "atomistic" way, by independent reproduction of a number of units and incorporation of these into the final phage particles. A number of problems may be raised, as to the nature of the self-reproducing units, the structure of the phage particle, and the relation of its mechanism of reproduction to that of other self-reproducing entities (genes, plasmagenes).

In the first place, each unit seems to react as a distinct photochemical entity in respect to ultraviolet quanta. The assumption of equal sensitivity. is very likely unjustified; a variation in sensitivity from locus to locus would tend to make the calculated number of loci per particle too small, since the most sensitive units have a greater chance of being lethally hit earlier in all infecting particles. This would result in less reactivation and apparently lower number of loci. Our estimates of the number of loci are therefore minima. It is interesting to notice that phage T4, which is about twice as resistant to ultraviolet than the related phages T2 and T6, also appears to have fewer loci. Its higher resistance seems, therefore, to be due

to actual absence of a number of loci rather than to absence of some particularly sensitive locus.

Our results with the small phages T1 and T7, where no reactivation was detected, do not prove that these phages do not possess a number of independently transferable loci. They only indicate that, if such loci occur, they are too few to be detected in our experiments, that is, fewer than 8 or 10. These phages are actually about 2.5 times more resistant to ultraviolet than phage T4.

As for the failure of particles inactivated by ionizing radiation (x-rays) to undergo reactivation, this may be interpreted as indicating, either spread of the lethal effect of each ionization (or group of ionizations) to a large number of loci, or, less likely, an ability of the x-rays to cause other changes than those produced by ultraviolet. A projected study with various monochromatic radiations may throw some light on this question. It is interesting to notice that Lea and Salaman,[10] extending earlier work on the rate of inactivation of phage by ionizing radiation,[11] and analyzing their results in terms of the density of ionization, recently concluded that the "sensitive zone" of a large phage particle may be resolved into about fourteen units (genes?). Because of the assumptions involved in their calculations, their results by themselves can hardly be considered as more than qualitative indication of a geometrically complicated organization of the radiation sensitive material of the phage particle. In the light of our experiments, however, the interpretation offered by Lea and Salaman appears quite plausible, at least in assuming a multiplicity of sensitive units.

The incorporation of discrete units into organized phage particles is not easy to visualize, in view of the complex structure and morphology of the latter.[12] We can imagine that each active unit impresses its specificity on a number of elements produced in excess inside the host cell, elements which represent the raw material then utilized to build more phage particles. The units carrying lethal mutations may be unable to mold the substrate in their own image and likeness.

It is plausible, although not yet experimentally demonstrated, that some of the transferrable units that can undergo lethal mutations are the same gene-like entities responsible for determination of the transferable characters studied by Delbrück and Bailey[6] and by Hershey[18].

The mechanism of transfer suggested for bacteriophages appears to differ from that of crossing-over by being a transfer of units by "infection" rather than an "exchange" of portions of gene strings. One might, however, conceive that excess gene copies similar to the copies of our units are also produced in the cell nucleus, but have no opportunity of being incorporated by "infection" into the chromosomal continuum because of a more rigid integration of the genes in the chromosome (needed to meet the requirements of the sexual process) than of the units in the virus particle. Excess pro-

duction of full or partial gene copies has been suggested both on the basis of cytological studies[13] and in connection with theories of gene action.[14]

The transfer mechanism appears to represent a novel, and, in its way, rather efficient means of effecting genetic recombination between virus particles.

Our results thus offer direct evidence for interpreting inactivation of viruses by radiation as due to lethal mutations, and strengthen the concept of a fundamental similarity between viruses and the genetic material of other organisms. They indicate that a virus particle may rather be comparable to a gene complex than to an individual gene. A virus particle can undergo a number of independent mutations,[2, 15] a property that may be shared by certain "gene complexes," defined as cross-over units resolvable into two independently mutable units.[16] The independent mutability of these subgenic units is, however, somewhat doubtful.[16] The virus particle might better be compared to a chromosome, although it behaves more as a unit toward x-rays than the latter. The self-reproducing components seem to possess in the virus particle a degree of solidarity intermediate between that of the components in the supposed gene complex and that of the genes in the chromosome.

There are obvious similarities between the transfer phenomenon here described and other phenomena in which a determinant of heredity is transferred into a new genetic complex, as in the case of type transformation in pneumococci.[17] Phage reproduction may be a particularly favorable material on which to analyze the mechanisms involved.

One interesting application is the possibility of analyzing differences between related but independent wild-type phage strains which can be "crossed" (like the T-even group) in terms of differences in the number of units that can be exchanged (shared loci). Preliminary work in this direction appears promising.

Summary.—Inactivation of large bacteriophages by ultraviolet light is due to lethal mutations at a number of different loci. Each of these loci appears to be independently transferable from one phage particle to others inside the same bacterium. The transfer of loci between irradiated inactive particles results in formation of active phage if the infecting particles, taken as a group, possess at least one copy of each locus in active form. The number of loci can be calculated for each phage, and is at least 30 to 50 for some of them. Phage growth appears to take place by independent reproduction of each of these "unit loci" inside the host cell.

[1] Delbrück, M., *Biol. Rev.* (Cambridge), **21**, 30 (1946).

[2] Hershey, A. D., *Genetics*, **31**, 620 (1946).

[3] Latarjet, R., and Wahl, R., *Ann. Inst. Past.*, **71**, 336 (1945).

[4] Luria, S. E., and Latarjet, R., *J. Bact.*, **53**, 149 (1947).

[5] Luria, S. E., and Delbrück, M., *Arch. Biochem.*, **1**, 207 (1942).

[6] Delbrück, M., and Bailey, W. T., Jr., *Cold Spring Harbor Symp.*, **11**, 33 (1946).

[7] Formula (1) was derived as follows:

Probability that a given locus receives no lethal mutation $= e^{-r/n}$.

Probability that a given locus receives a lethal mutation $= 1 - e^{-r/n}$.

Probability that a given locus receives a lethal mutation in all of k particles $=$ $(1 - e^{-r/n})^k$.

Probability that a given locus does not receive a lethal mutation in all of k particles $= 1 - (1 - e^{-r/n})^k$.

Probability that none on n loci receives a lethal mutation in all of k particles $=$ $y_k = [1 - (1 - e^{-r/n})^k]^n$

[8] It should be noticed that x (average number of phage particles adsorbed per bacterium) is different from k (actual number of phage particles adsorbed by a given bacterium). The proportion of cells with k particles is $(x^k/k!) e^{-x}$.

[9] These results differ from earlier observations[5] of interference between inactive and active particles of the same phage. The older experiments, however, were done under conditions not comparable with those discussed here.

[10] Lea, D. E., and Salaman, M. H., *Proc. Royal Soc.*, B, **133**, 434 (1946).

[11] Wollman, E., Holweck, F., and Luria, S. E., *Nature*, **145**, 935 (1940). Luria, S. E., and Exner, F. M., these Proceedings, **27**, 370 (1941).

[12] Luria, S. E., and Anderson, T. F., *Ibid.*, **28**, 127 (1942).

[13] See Schultz, J., *Colloid Chemistry*, **5**, 819 (1944).

[14] See Emerson, S., *Ann. Missouri Bot. Garden*, **32**, 243 (1945).

[15] Luria, S. E., *Genetics*, **30**, 84 (1945).

[16] Stadler, L. J., *Ibid.*, **31**, 377 (1946).

[17] See McCarty, M., *Bact. Rev.*, **10**, 63 (1946).

[18] Hershey, A. D., *Cold Spring Harbor Symp.*, **11**, 67 (1946).

NUTRITIONAL LIFE HISTORY AS INFLUENCED BY DIETARY ENRICHMENTS. I.*

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Starting with a basal diet of natural foods which is adequate in the sense and degree that rat families are thriving upon it in the 63rd generation, the effects of enriching the diet (*a*) with protein in the form of poultry meat, and (*b*) with added calcium also, are being studied.

These enrichments in protein increase the rate of growth and result in earlier puberty. With females, however the records from puberty to middle age are variable when the dietary is enriched in protein food only. They raise the question whether the more rapid growth and earlier sexual