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# ADVANCES IN BIOLOGICAL AND MEDICAL PHYSICS

*Edited by*

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*Assistant Editor*

THOMAS L. HAYES

University of California, Berkeley, California

*Editorial Board*

H. J. CURTIS · L. H. GRAY

A. K. SOLOMON · BO THORELL

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# GENETIC AND PHYSIOLOGICAL EFFECTS OF THE DECAY OF INCORPORATED RADIOACTIVE PHOSPHORUS IN BACTERIAL VIRUSES AND BACTERIA

By Gunther S. Stent and Clarence R. Fuerst

Virus Laboratory, University of California, Berkeley, California  
Ontario Cancer Institute, Toronto, Ontario, Canada

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## I. INTRODUCTION

Who is there today that has not heard that deoxyribonucleic acid (DNA) is the genetic material into which hereditary determinants are inscribed? Recondite for three-quarters of a century following its discovery by Miescher in 1871, DNA is now familiar to everyone. For the elucidation of the structure and function of this substance promises to bridge the gap between chemistry and genetics, the two complementary aspects of living matter.

Although the combination of the basic DNA constituents, phosphoric acid, deoxyribose, adenine, guanine, cytosine, and thymine, into nucleotides was established long ago, it was realized only relatively recently that DNA molecules are, in fact, polymers of very high molecular weight, each molecule containing more than  $10^4$  nucleotidic units joined together through phosphate diester bonds linking successive deoxyribose molecules. The actual molecular structure of DNA was not recognized until 1953, when Watson and Crick first showed that DNA consists of two helically intertwined polynucleotide chains laterally held together by a pair of hydrogen bonds between a complementary pair of purine and pyrimidine residues on opposite chains, a development which engendered the present flowering of so-called molecular biology.

While its connection with the hereditary determinants was long suspected, the identity of DNA with the carrier of genetic continuity was at last clearly revealed by two great discoveries. The first was the finding of Avery *et al.* (1944) that pure deoxyribonucleate extracted from one strain of bacteria can impose some of the hereditary characters of the donor on the progeny cells of a receiver strain, showing that the bacterial genome is carried in the bacterial DNA. The second was the finding of Hershey and Chase (1952) that infecting bacterial virus particles inject their DNA, but very little of their protein, into the interior of the bacterial host cell at the outset of the reproductive process, showing that the viral genome is likewise carried in the viral DNA. In its role as the hereditary material, the bacterial or viral DNA must fulfill both *genotypic* and *phenotypic* functions, that is to say, it must replicate in order to provide the genetic substance for progeny cells or viruses, and it must induce, or preside over, the synthesis of various other biochemical materials, in particular of enzyme pro-



teins, to give physical expression to the specific hereditary information which it carries.

In order to find out how the DNA accomplishes its two tasks, many biochemical, genetic, and radiobiological methods have been applied to the study of the growth and reproduction of bacteria and bacterial viruses (or bacteriophages). Very prominent among these methods has been the use of radioactive tracers, especially of the phosphorus isotope P<sup>32</sup>, which can be readily incorporated into the phosphate diester bonds of the DNA polynucleotide chains.

Radiophosphorus is a particularly suitable tracer isotope for bacteriophage studies since these viruses are composed approximately half of protein and half of DNA, the latter constituting the only, or at least the principal, phosphorylated component of the particle. P<sup>32</sup>-labeled bacteriophages, therefore, harbor the radioactivity mainly in their DNA. In such tracer experiments, the presence of the radioisotope is generally detected by placing large numbers of these small bodies, or extracts obtained from their mass cultures, in front of conventional radiation counters. In order to answer certain fundamental questions, however, it is some times necessary also to measure the radiophosphorus content of *individual* viruses or cells; for this purpose ordinary counters cannot be employed. For instance, at even the highest practicable specific activities, no more than 3000 P<sup>32</sup> atoms, producing less than six disintegrations per hour, can be incorporated into a single phage. Nevertheless, it has proven possible recently to estimate the radiophosphorus content of individual labeled bacteriophages and bacteria *autoradiographically* by embedding them in highly sensitive "nuclear" emulsions and counting the number of  $\beta$ -electrons seen to have emanated from each particle after a known exposure period (Levinthal, 1955, 1956; Forro, 1957).

In 1950, however, a radically novel idea of estimating the radiophosphorus content of single microorganisms was conceived by Hershey, Kamen, Kennedy, and Gest: namely, that the presence of radiophosphorus might be detected not only through the emitted radiation but also through the *lethal effects* produced by the decay of the radioactive atoms within the DNA molecules in which they happened to be incorporated. For if each decay of one of its incorporated P<sup>32</sup> atoms has a certain probability of killing the organism, then the rate of inactivation of a labeled population reflects the isotope content of the individuals. As we endeavor to show in this review, the method of Hershey, Kamen, Kennedy, and Gest has not only proven highly suitable for the purpose for which it was conceived, but it has also found valuable application in radiobiological studies on the structure and function of the genetic material of bacterial viruses and bacteria.

## II. INACTIVATION OF BACTERIAL VIRUSES

A. Instability of Highly  $P^{32}$ -Labeled Virus Particles

It was discovered by Hershey *et al.* (1951) that bacteriophage particles labeled with radiophosphorus are unstable. In their experiments, Hershey *et al.* inoculated bacteriophages into sensitive bacterial cultures growing in a medium of high specific  $P^{32}$  activity, so that the progeny phage particles produced by this infection likewise contained a high level of radioisotope among the phosphorus atoms of their DNA molecules. When dilutions of such populations of labeled phages were kept in the refrigerator and assayed daily for their infectivity, it was found that as the radioactive atoms decayed, a progressively decreasing fraction of the phage population retained the ability to reproduce itself, i.e., to form a plaque on the sensitive bacterial indicator strain. This loss of infectious titer occurred even in highly dilute suspensions of labeled phage, indicating that the inactivation of one phage particle was not due to the radiation emitted by radioisotope contained in other phages or in the medium but was the consequence of the disintegration of its own atoms of  $P^{32}$ .

Figure 1 presents the survival of a stock of highly  $P^{32}$ -labeled T2 phage, as well as that of a nonlabeled T2 control population, stored at 4°C. and

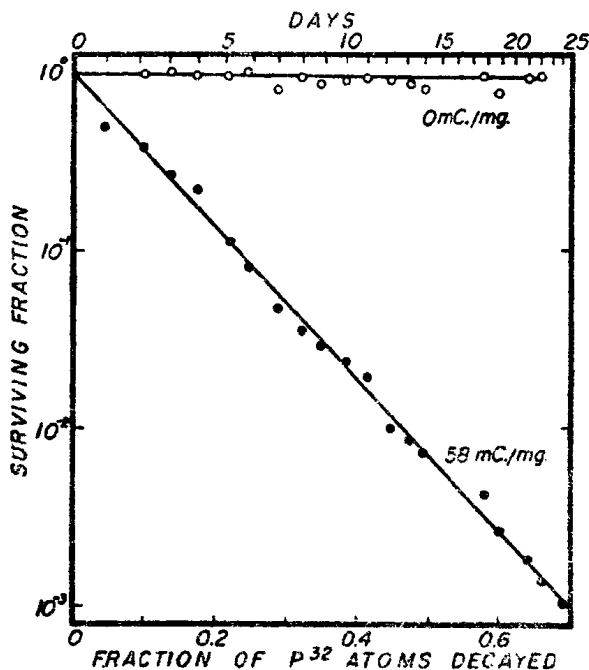


FIG. 1. Survival of labeled and nonlabeled stocks of T2 bacteriophage at 4°C. Data replotted from Hershey *et al.* (1951).

periodically assayed over several weeks. It is seen that whereas the non-labeled phages are stable under these storage conditions, the highly P<sup>32</sup> labeled population is markedly unstable, the logarithm of the number of surviving individuals falling linearly with the number of P<sup>32</sup> atoms having decayed up to the time of assay.

The simple exponential nature of the inactivation kinetics establishes two important points. First, it indicates that a single P<sup>32</sup> disintegration can inactivate a phage particle, for if more than one disintegration were required for inactivation, the survival curve would be convex. Secondly, it shows that the particles in the population do not differ in their susceptibility to inactivation by P<sup>32</sup> decay, for if some particles were more sensitive than others, the survival curve would be concave. When Hershey *et al.* compared the inactivation of phage stocks grown on media labeled with different levels of P<sup>32</sup>, they found that more highly labeled lysates lost their titer faster than less highly labeled lysates, the rate of inactivation (i.e., the slope of the exponential survival curve) being proportional to the specific radioactivity of the growth medium.

These observations led Hershey *et al.* to express the rate of inactivation of radioactive virus particles as

$$dS/dt = -\alpha N^* \sigma S \quad (1)$$

where  $S$  is the titer of infectious phage particles per milliliter,  $t$  the number of days,  $\alpha$  the efficiency of killing per atomic disintegration,  $N^*$  the number of radioactive phosphorus atoms per particle, and  $\sigma$  the fractional decay of P<sup>32</sup> per day. Integration of Eq. (1) and introduction of more suitable parameters leads to the survival equation

$$\log_{10} s = -1.48 \times 10^{-6} \alpha A_0 N (1 - e^{-\sigma t}) \quad (2)$$

where  $s$  is the fraction of particles surviving,  $N$  the total number of phosphorus atoms per phage particle, and  $A_0$  the specific radioactivity of the growth medium in mC./mg. P. This equation correctly predicts that a plot of  $\log_{10} s$  vs.  $1 - e^{-\sigma t}$  (the fraction of P<sup>32</sup> atoms decayed) is a straight line whose slope is proportional to the specific radioactivity of the growth medium.

### B. The Efficiency of Killing

The slope of the survival curve of P<sup>32</sup>-labeled phages is thus a function of the parameters  $A_0$ ,  $N$ , and  $\alpha$ . Since the values of  $A_0$  and  $N$  can be established directly by radiochemical analysis,  $\alpha$ , the efficiency of killing per P<sup>32</sup> disintegration, can be calculated from the slope of the survival curves. Hershey *et al.* found on the basis of their data that this efficiency of killing

was approximately 0.1 in T2 and T4 phage, i.e., that about one out of every ten disintegrations kills the phage particle in which it occurs.

The inactivation by decay of a variety of phage strains has been studied in the meantime, and the results of some of this work have been summarized in Table I. As can be seen there, the strains so far investigated differ rather markedly from one another in size, chemical composition, and phosphorus content. Their biological behavior, furthermore, particularly their genetics and interaction with the host cell, is likewise subject to rather profound differences (Stent, 1958). Nevertheless, it is evident that the first eight phage types listed in Table I are very similar in their sensitivity to  $P^{32}$

TABLE I  
*Inactivation of Various E. coli Phage Strains by  $P^{32}$  Decay*  
*Evaluation of the Parameters of the Survival Equation*  
 $\log_{10} s = -1.48 \times 10^{-6} \alpha A_{40} N (1 - e^{-\epsilon t})$  at 4°C.

1 Bacteriophage strain	2 Dimensions of head structure (m $\mu$ )	3 DNA base ratios (A+T/G+C)	4 N (Atoms per phage)	5 N $\alpha$ (Lethal atoms per phage)	6 $\alpha$	References			
						Col. 2	Col. 3	Col. 4	Col. 5
T2 <sup>a</sup>	95 × 65 <sup>a</sup>	2.2 <sup>a</sup>	3.9 × 10 <sup>5</sup>	4.5 × 10 <sup>4</sup>	0.12	(13)	(14)	(4)	(10)
T4 <sup>a</sup>	95 × 65 <sup>a</sup>	2.2 <sup>a</sup>	5.0 × 10 <sup>5</sup>	4.3 × 10 <sup>4</sup>	0.09	(13)	(14)	(5)	(5)
T5	65 <sup>a</sup>	1.6	3.5 × 10 <sup>5</sup>	4.2 × 10 <sup>4</sup>	0.12	(13)	(15)	(10)	(10)
T1	50 <sup>a</sup>	1.1	1.4 × 10 <sup>5</sup>	1.7 × 10 <sup>4</sup>	0.12	(13)	(17)	(10)	(10)
T3 <sup>b</sup>	47 <sup>f</sup>	1.0	2 × 10 <sup>5</sup>	1.3 × 10 <sup>4</sup>	0.07	(13)	(2)	(10)	(10)
T7 <sup>b</sup>	47 <sup>f</sup>	1.1	1.6 × 10 <sup>5b</sup>	1.6 × 10 <sup>4</sup>	0.10	(13)	(7)	(7)	(10)
$\lambda$	Like T5	1.0	2.3 × 10 <sup>5</sup>	1.5 × 10 <sup>4</sup>	0.07	(6)	(9)	(10)	—
P22 <sup>d</sup>	50-60 <sup>f</sup>	1.0	1.4 × 10 <sup>5</sup>	1.2 × 10 <sup>4</sup>	0.09	(16)	(16)	(3)	(3)
$\phi$ X174 <sup>c</sup>	25	1.2	5.1 × 10 <sup>3i</sup>	5.7 × 10 <sup>3</sup>	1.1	(1)	(8)	(8)	(11)
S13 <sup>c</sup>	—	—	5.1 × 10 <sup>3j</sup>	5.5 × 10 <sup>3</sup>	1.1	—	—	—	(12)

<sup>a,b,c</sup> Serologically related.

<sup>d</sup> A *Salmonella* phage.

<sup>e</sup> Elongated hexagon.

<sup>f</sup> Hexagon.

<sup>g</sup> Thymine replaced by 5-hydroxymethylcytosine. Glucose also present.

<sup>h</sup> Thirty per cent in material other than DNA.

<sup>i</sup> Inferred from DNA content and molecular weight of phage.

<sup>j</sup> Assumed value.

REFERENCES: (1) Hall *et al.*, 1959; (2) Fraser and Nakamura, 1953; (3) Garen and Zinder, 1955; (4) Hershey *et al.*, 1953; (5) Hershey *et al.*, 1951; (6) Kellenberger, 1957; (7) Lunan and Sinsheimer, 1956; (8) Sinsheimer, 1959; (9) Smith and Siminovitch, 1953; (10) Stent and Fuerst, 1955; (11) Tessman, 1959; (12) Tessman *et al.*, 1957; (13) Williams, 1953; (14) Wyatt and Cohen, 1952; (15) Wyatt and Cohen, 1953; (16) Zinder, 1955; (17) Creaser and Taussig, 1957.

decay, in that the efficiency of killing per disintegration is in the vicinity of 0.1 in all strains. Only in the last two phage types listed is the efficiency of killing seen to be much greater, a fact which we must ask the reader to ignore temporarily in this discussion. Both the similarity in efficiency of killing in the first eight as well as the great difference in the P<sup>32</sup> sensitivity of the last two strains provide an important clue to the nature of the mechanism of inactivation of phage particles by radioactive decay.

### C. The Mechanism of Lethal Action

1. *Relative Importance of Transmutation and Radiation.* When a P<sup>32</sup> atom disintegrates, a beta electron of maximum kinetic energy 1.7 Mev. is emitted, and the nucleus of the sulfur atom that is produced sustains a recoil which may amount to as much as 80 ev. *A priori*, inactivation of P<sup>32</sup>-labeled phages could, therefore, be due either to the noxious effects of the internal radiation attending radioactive decay or to some fatal event engendered by the fate of the transmuted atom itself.

Hershey *et al.* (1951) already evaluated the possible importance of the internal radiation in the inactivation of phage T4. On one hand, they showed by means of simple calculations based on: (a) the average track length within the phage of beta particles originating in randomly situated internal disintegrations (Lind, 1921); (b) the number of ion pairs produced per unit length of electron track; and (c) the rate of inactivation of P<sup>32</sup>-labeled T4 phages, that if such radiation-induced ionizations are the principal cause of death, the efficiency of killing *per ionization* would have to be of the order of 0.3. On the other hand, Hershey *et al.* also measured directly the efficiency of killing per ionization by suspending nonradioactive T4 phages in a medium containing a high level of inorganic P<sup>32</sup> atoms. In that case, the survival of the phage bombarded by external beta electrons is given by

$$\log_{10} s = \frac{-6.9 \times 10^{16}}{2.3\sigma} m_0 a V (1 - e^{-\sigma t}) \quad (3)$$

where  $6.9 \times 10^{16}$  is the rate of ionization per day per millicurie of radioactivity,  $m_0$  is the initial radioactivity in millicuries per milliliter,  $a$  is the efficiency of killing per ionization within  $V$ , the volume of the phage particle. It was found in this way that the efficiency of killing, by ionization produced within the phage by beta electrons originating from the decay of *external* P<sup>32</sup> atoms, was only 0.009. This low efficiency of killing per ionization shows therefore that at most 1/30 of the lethal effects of decay of internal P<sup>32</sup> atoms can be due to internal ionization and must, instead, be caused by some "short-range" consequence of the transmutation of phosphorus to sulfur.

This inference has gained ample support, in the meantime, from other studies. The observation, for instance, that the efficiency of killing per P<sup>32</sup>

disintegration is the same for a number of phage strains of different particle size (Table I) speaks against the view that radiation effects are the principal cause of phage death. For each disintegration would produce a greater number of ion pairs in the larger phages and, hence, should have a greater efficiency of killing in such strains. Other relevant evidence will be presented in later sections. It is to be concluded, therefore, that beta particles emitted in  $P^{32}$  decay are not an important cause of phage death.

2. *Interpretation of the Lethal Effects in Terms of the Structure of DNA.* Most, if not all, of the phosphorus of bacterial virus particles is nucleic acid-phosphorus; the nucleic acid in every strain that has been subjected to chemical analysis thus far has been found to be of the deoxy type (DNA). Thus, lethal and nonlethal  $P^{32}$  disintegrations occur almost exclusively in DNA, the germinal substance of the extracellular phage that initiates intracellular phage development (Hershey and Chase, 1952; Hershey, 1957).

An interpretation of the lethal effects of  $P^{32}$  decay was advanced by Kamen (1950), who evaluated the possible disruptive effect of nuclear recoil on the phosphate ester bonds which link nucleoside residues in DNA. If reasonable values are assigned to the energy of the P—O bonds, to the energy of the recoil, and to the mass of the material affected, one finds that almost every disintegration should lead to the rupture of an ester bond. Furthermore, replacement of phosphorus by sulfur might of itself lead to bond cleavage, owing to the probable instability of the sulfate diester that would result even if both S—O bonds survived the effects of nuclear recoil (Stent and Fuerst, 1955). It seems probable, therefore, that every  $P^{32}$  disintegration causes an interruption of a polynucleotide chain.

Thus, while all  $P^{32}$  disintegrations are likely to have the same primary effect, they are clearly differentiated into two types. One, the majority, has no effect whatsoever on the viability of the phage, and the other, a minority, kills the phage in which it takes place. In order to explain this difference, Hershey *et al.* suggested that the nucleic acid of the phage might be composed of "essential" and "nonessential" fractions. In other words, the supposition was made that every  $P^{32}$  disintegration is lethal if it occurred in the "essential" DNA fraction, which accounts for approximately 10% of the nucleic acid in the particle. This notion loses much of its credibility in the light of the comparative studies with various phage strains (Table I). For it would be necessary to assume that in all of the phages "essential" and "nonessential" fractions are of the same relative size, although the particles of some strains contain three times more DNA than the particles of other strains. It will become more apparent when the environmental effects on phage inactivation are discussed that the idea of an heterogeneity of the phage DNA does not provide an adequate explanation of the low efficiencies of killing per  $P^{32}$  disintegration in these strains.

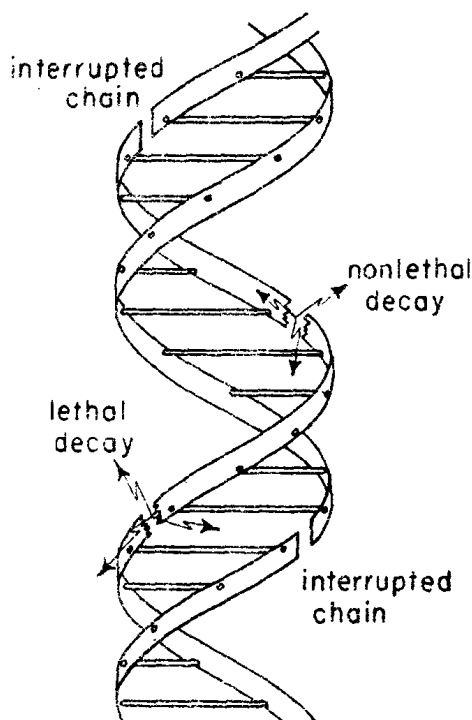


FIG. 2. Schema of the Watson-Crick structure of DNA and of the lethal effects of P<sup>32</sup> decay. The two ribbons symbolize the two phosphate sugar chains, and the horizontal rods represent the base pairs holding the chains together through a pair of hydrogen bonds.

It seemed much more probable that the nature of the lethal action of P<sup>32</sup> decay is to be explained in terms of the molecular structure of DNA itself, presented diagrammatically in Fig. 2 (Watson and Crick, 1953a). The two paired ribbons represent the two polynucleotide chains of the DNA molecule, which are maintained in the configuration of a double helix by hydrogen bonds, shown as horizontal bars, between purine and pyrimide bases of the opposite chains. The phosphorus atoms are situated in diester linkage between nucleoside residues in each chain, so that a phosphorus atom can be thought of in this representation as being part of the ribbon between two neighboring cross-bars.

Let us now consider the inactivation by P<sup>32</sup> decay of a phage like T2, whose DNA appears to be in the two-stranded structure (Wilkins *et al.*, 1953; Thomas, 1959). The P<sup>32</sup> atoms in the polynucleotide chains will be rather far apart in terms of the number of intervening nucleoside residues since, even in the most radioactive T2 particles obtainable, no more than 1-2% of the phosphorus atoms can be of the P<sup>32</sup> isotope. Now, although every P<sup>32</sup> disintegration might cleave the polynucleotide chain in which it

occurs, it seems unlikely that breakage of only one chain would have a marked effect on the over-all structural integrity of the macromolecule, for, as shown in Fig. 2, the hydrogen bonds which hold the two chains together still ought to keep in position the two fragments of an interrupted chain (Dekker and Schachman, 1954). Since only 0.1 of all the  $P^{32}$  disintegrations are of the lethal type, there is no alternative but to conclude that this low efficiency of killing means that the DNA molecule retains its biological activity even after some interruptions of its polynucleotide chains have been generated by radioactive decay. An event secondary to the disruption of the phosphate diester bonds must then attend the lethal disintegrations, for which the most reasonable hypothesis appeared to be *a complete cut of the DNA double helix*.

Since the survival curves of radioactive phage populations are exponential, it would then follow that on occasion a *single*  $P^{32}$  disintegration can cut both chains, in contrast to scissions caused by cooperation of two or more  $P^{32}$  disintegrations which happen to have occurred in near apposition on opposite chains and which should lead to "multiple-bit" survival curves. It seems likely that the lethal disintegrations are those in which the recoil of the disintegrating nucleus in one polynucleotide chain is sufficiently energetic and suitably oriented toward the second chain to cause a break there also (Stent, 1953b; Stent and Fuerst, 1955).

The present hypothesis identified the disintegrations which destroy the biological activity of the phage as those which depolymerize DNA macromolecules, and therefore predicts that the efficiency of killing and of molecular cleavage is the same. This prediction has recently found confirmation in a study by Thomas (1959) of the effect of radioactive decay on the molecular weight of phage DNA. It can be shown by autoradiography that about 40 % of the DNA of T2 particles is released by osmotic shock from the phage head as a single aggregate, while the remainder of the DNA is released in fragments too small to be detected by this technique (Levinthal, 1955; Levinthal and Thomas, 1957). Thomas extracted the DNA of highly  $P^{32}$ -labeled T2 phages after various amounts of radioactive decay and then estimated by autoradiography the number of phage particles which still release an intact DNA aggregate. This experiment showed that fewer and fewer large aggregates are found the more decay had taken place. From the rate of disappearance of these aggregates, it could be inferred that about one in every five  $P^{32}$  disintegrations causes a reduction in size. Hence there exists rough quantitative agreement between the fraction of disintegrations which is lethal and the fraction which causes a cut of the DNA macromolecule, in support of the view that both fractions pertain to the same basic event. Thomas also examined by this method the survival of the large DNA aggregate, if autoradiography is undertaken only after heating the



plasmolyzed phage suspensions briefly to 95°C. While such heating of non-labeled phage DNA, or of P<sup>32</sup>-labeled phage DNA before decay, causes no serious reduction in the number of observable DNA aggregates, it was found that heated aggregates vanish with decay at a much greater rate, as if under these conditions *every* disintegration destroyed the integrity of the aggregate in which it occurred. This finding supports the view expressed above that all of the nonlethal P<sup>32</sup> disintegrations cleave single polynucleotide chains. For although such single cleavage might leave the macromolecule structurally intact and viable under ordinary conditions, the two strands of the DNA duplex should be more readily dissociated and the molecule depolymerized by heat after they have been occasionally interrupted along their length.

#### D. Effects of Temperature

In the experiments described up to this point, the instability of labeled bacteriophages was always studied at 4°C. When radioactive virus particles were stored at a different temperature, however, it was found that their P<sup>32</sup> decay sensitivity was now changed. As Fig. 3 shows, the rate of

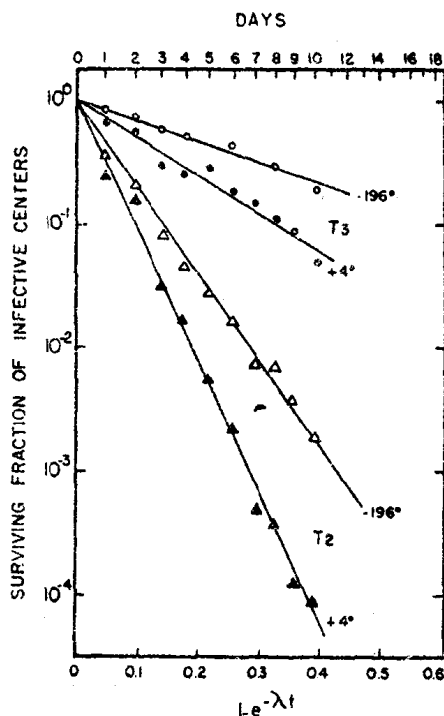


FIG. 3. Survival of P<sup>32</sup>-labeled stocks of T2 and T3 bacteriophage ( $A_0 = 160$  mC./mg.) at 4°C. and at -196°C. (Stent, 1953a).