EFFECTS OF DECAY OF INCORPORATED H^3-THYMIDINE ON BACTERIA

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ABSTRACT The killing efficiency due to the decay of incorporated H^3-thymidine in three mutants of *E. coli strain* 15: 15_{T-}, 15_{T-L-}, and 15_{T-U-} has been determined. This efficiency is comparable to that previously determined by others for P^{32} decay. The killing efficiency has been determined as a function of H^3-thymidine specific activity, storage media and storage temperature. We have observed a latent killing effect that causes lethality under certain conditions. The kinetics of latent killing have been examined at several temperatures. Finally, mutation production induced by H^3-thymidine decays was shown to occur. The results are consistent with the idea that inactivation and mutations may be caused by a process in the nuclear transmutation that is not associated with β-particle ionization damage.

INTRODUCTION

Since the discovery by Hershey, Kamen, Kennedy, and Gest (1951) that the decay of radiophosphorus incorporated into the DNA of bacterial viruses produced death[1] P^{32} suicide has become widely used as a research technique (see Stent and Fuerst, 1960). Stent and Fuerst (1955) and Fuerst and Stent (1956) have studied the effects of P^{32} decay on several biological functions of bacterial viruses and bacteria. Kaudewitz, Vielmetter, and Friedrich-Freska (1958) have shown that P^{32} decays result in mutation production.

It is possible that data resulting from decays of incorporated H^3-labeled thymidine in bacteria may prove even more interesting for the following reasons. (1) The label is incorporated exclusively into the DNA and moreover into the "genetic part" of the DNA. (2) The recoil nucleus, He^3, is biologically unimportant and chemically unreactive. (3) The average energy of the recoil nucleus is approximately 1 ev which is probably too low to rupture covalent bonds. (4) The decay of H^3 from thymidine perhaps involves only a subtle change in a single strand of DNA as contrasted

[1] This effect has come to be known as suicide.

with the main chain scission that is thought to occur after a lethal P^{32} decay. However the β-particle accompanying H^3 decay has a maximum energy of only 18 kev and, therefore, the possibility of radiation damage due to ionizations along the path of the β-particle must be considered.

It is possible that DNA function could be impaired by the removal of a single H atom and the resulting molecular rearrangements that may take place at a specific locus in the thymidine moiety of DNA. Thus, if it can be shown that decays from H^3-thymidine produce their effects by a mechanism other than radiation damage it may be a valuable tool for the study of DNA functions, especially those concerned with mutation production. With this in mind we began a study of some effects of decay from incorporated H^3-thymidine in various mutants of *E. coli* strain 15. We have shown that mutations are induced as a result of H^3 decay. We have also determined the killing efficiency (α_{H^3}), the probability of producing a lethality by a single H^3 decay, for three mutants of strain 15, under a variety of experimental conditions. A preliminary account of this work was given to the Radiation Research Society (Person and Lewis, 1959). The killing efficiency is comparable to that observed for P^{32} decays in bacteria (Fuerst and Stent, 1956) and for H^3-thymidine decays in bacteriophage (Cairns, 1961). However, our value for the killing efficiency differs from that reported by Apelgot and Latarjet (1962) using H^3-thymidine decays in bacteria. Finally we have observed latent killing in labeled cultures that have been stored at −78°C.

MATERIALS AND METHODS

Stock Cultures and Growth Medium. Three mutants of strain 15 were used in these experiments: 15_{T-}, which is thymidine-deficient; 15_{T-L-}, thymidine- and leucine-deficient; 15_{T-U-}, thymidine- and uridine-deficient. Cultures of these mutants were obtained from Dr. F. Forro, Jr., Yale University. Stock cultures were maintained on nutrient agar. All cultures for experimental use were grown in A-1 minimal medium: 2 gm NH_4Cl, 6 gm anhydrous Na_2HPO_4, 5 gm NaCl, 0.115 gm Na_2SO_4, 0.340 gm $MgCl_2 \cdot 6H_2O$, 4 gm glucose in a liter of water. The medium is supplemented according to the mutant deficiency and at concentrations that are in excess to avoid limiting growth. Thymidine is added to a final concentration of 4 μg/ml; leucine, 40 μg/ml; uridine, 90 μg/ml.

Labeling Procedure. The appropriate mutant of *E. coli* 15 was inoculated from a stock culture into supplemented A-1 and grown with aeration at 37°C to a titer of 1-2 × 10^8/ml.[2] It was then diluted 100- to 200-fold in prewarmed A-1. Approximately 4 ml aliquots of this dilution were added to either 0.05 ml of an aqueous solution of cold thymidine or 0.1 ml of H^3-thymidine.[3] The final concentration of thymidine in both cultures was 2 μg/ml. The specific activity of H^3-thymidine generally used was 3.0 c/mmole. Supplements of leucine or uridine were added if required. The cultures were incubated at 37°C with aeration and cell counts were made at 1 to 2 hour intervals on both the control and radioactive culture. The doubling time was approximately 75 min-

[2] Cell counts were made with a Coulter cell counter, Coulter Electronics, Chicago.

[3] Obtained from Schwarz Bioresearch, Orangeburg, New York.

utes in each case. Since care was taken to prewarm the media, supplements, and growth flasks, a lag phase was not induced at the onset of the labeling procedure. The cultures were incubated until the titer had increased from 10^7 to 5×10^8/ml = 50-fold, or between 5 and 6 cell divisions.

Preparation of Cultures for Cell Counts and H^3 Counts. At the end of the labeling period the cultures were gently filtered onto a 0.45 micron Millipore filter pad[4] and washed repeatedly with A-1 medium. Radioactive counting of the final wash demonstrated that labeled medium had been removed. The coli were resuspended in the original volume of A-1. Aliquots were removed for final cell counts and H^3 counts so that the number of H^3-thymidine molecules incorporated/cell may be computed. The suspensions were then stored at the temperatures indicated.

H^3-Thymidine Decay. Cultures were stored in 0.8 per cent nutrient broth, A-1, or in A-1 containing glycerol at a final concentration of 10 per cent. Exogenous H^3-thymidine was added to the control culture at the time of storage in an amount in excess of that incorporated into the radioactive culture. This external source of H^3 decays was without effect.

Cell suspensions were stored at −78°C by pipetting 0.1 ml aliquots of control and radioactive cultures into small test tubes precooled to −78°C in a dry ice-isopropyl alcohol mixture.

H^3 Counting. All H^3 counting was done using a Tri-Carb liquid scintillation counter.[5] Sample volumes of 0.05 ml of whole coli were added to 20 ml of the following scintillation mixture: 4 gm PPO-diphenyloxazole and 15 mg of POPOP-1, 4-bis-2-(5 phenyloxazolyl)benzene dissolved in 77 per cent toluene and 23 per cent absolute ethanol. The counter efficiency was determined by the use of a H_2^3O standard obtained from the National Bureau of Standards. The counter efficiency, about 18 per cent, was measured repeatedly. Unlabeled bacteria from control cultures were added to the standards to eliminate quenching errors due to the presence of foreign organic matter.

The following experiment indicated that H^3-thymidine incorporated into bacteria reduced the counter efficiency by about one-half. H^3-thymidine was added to a culture of 15_{T-}, freshly diluted from a parent culture, to give a final thymidine concentration of 0.2 μg/ml. This concentration will limit growth at approximately 2×10^8/ml. The culture was incubated at 37°C and aliquots removed at intervals for H^3 counts until the culture attained limiting growth. H^3 counts were made directly on these samples without the usual filtration procedure. Any reduction in H^3 counts of the sequential samples is due to the label being incorporated into the bacteria. The total amount of H^3 remains constant with time, only the distribution between the medium and cells changes. H^3 counts in later samples were reduced to 43 per cent of those taken at the beginning of the incubation period. It is necessary to stop this experiment at the onset of limited growth because abnormally large cells begin to appear at this time. A culture of *E. coli* B was used as a control in this experiment as it is known that *E. coli* B converts thymidine to thymine at the surface of the coli (Setlow and Boyce, 1962) and thymine is incorporated only with difficulty (Crawford, 1958). Sequential H^3 counts from this culture remained constant with time.

Plating Procedure. Dilutions of cultures stored for various times were generally made in 0.8 per cent nutrient broth and plated for viable cells on Difco nutrient agar plates. To assay for sublethal mutations cells surviving storage at −78°C were plated

[4] Millipore Filter Corp., Bedford, Massachusetts.

[5] Packard Instrument Co., LaGrange, Illinois.

on EMB agar and scored if the colonies were white or white-sectored. Confirmation consisted of subculturing twice on EMB agar. These were recorded as mutants from lactose to non-lactose fermentation.

Estimation of Errors in the Determination of the Killing Efficiency. The killing efficiency, α_{H^3}, is calculated from the slope of the survival curve, the decay constant, and the number of H^3-thymidine labels/cell (see Results). Errors in the survival curve data and in the determination of labels/cell, therefore, represent the possible sources of errors in the determination of the killing efficiency.

Cell and H^3 counts were calculated from measurements on the labeled cell suspensions. The number of labels/cell (N^*) is the ratio of labels/ml to cells/ml. The cell counts were made using a Coulter counter. Generally 5 samples were counted in duplicate giving about 10^5 cells counted for each determination of N^*. The bacteria titer determined from colony-forming ability, measured immediately after labeling, was generally about 75 per cent of the cell count for both control and labeled cultures. In the liquid scintillation counting approximately 10^5 net counts were recorded per sample. Quenching of the liquid scintillation fluid would tend to give lowered counting rates, but the addition of unlabeled bacteria to the counting standards should have eliminated this possible source of error. N^* was usually determined twice in any experiment: once as soon as labeling was completed and again 2 days later. For both cell and H^3 counts we conclude that the largest sources of errors were pipetting errors. The minimum volumes pipetted were 0.05 ml and the errors in this measurement were at most ± 10 per cent. Since this occurs once for cell counts and once for H^3 counts we estimated that the determination of N^* is accurate to within ± 20 per cent. We estimate the possible error in the slope of the survival curve to be ± 10 per cent. Therefore, it seems to us that the determination of the killing efficiency cannot be in error by more than ± 30 per cent.

RESULTS

The Efficiency of Killing (α_{H^3}). The differential equation, first given by Hershey, Kamen, Kennedy, and Gest (1951) governing the rate of loss of surviving bacteria with time is

$$-\frac{dS}{dt} = \alpha\lambda N^* S \tag{1}$$

where S refers to the number of surviving bacteria at any time t, N^* is the number of radioactive atoms in each bacterium at time t, λ is the decay constant for the radioactive species used, and α is the probability of a lethal event being produced by a single nuclear transmutation. Since α reflects the lethal yield per decay it has come to be known as the killing efficiency.

N^*S is the total number of labeled molecules in a bacterial population of S cells at time t and λN^*S is the number of decays/unit time in that population. Therefore, the right-hand side of equation (1), $\alpha\lambda N^*S$, represents the rate of decrease of survivors with time.

Equation (1) cannot be integrated as it stands because N^* is a function of time. However, N^* may be considered constant for H^3 decay because $\lambda = 1.52 \times 10^{-4}$/day and our experiments are completed within 30 days. Thus, equation (1) may be

integrated directly and yields $\ln S/S_0 = -\alpha_{H^3}\lambda N^* t$ or $S/S_0 = \exp(-\alpha_{H^3} N^* \lambda t)$ where α_{H^3} refers to the killing efficiency for H^3-thymidine decay. This equation is one typical of "single hit" processes and the assumptions involved in single hit kinetics are therefore inherent in the differential equation. If the survival data obey this relationship then a plot of $\ln S/S_0$ as a function of time exposed to radioactivity will yield a straight line of slope $-\alpha_{H^3}\lambda N^*$. This is observed. Since λ is a constant and N^* may be determined experimentally by determining the number of H^3-thymidines and the number of *E. coli* cells in an aliquot of a labeled population, the killing efficiency, α_{H^3}, may be calculated. This procedure was used in determination of α_{H^3} in the following experiments.

Killing Efficiency, α_{H^3}, for 15_{T-}, 15_{T-U-}, 15_{T-L-}. Fig. 1 is a semilogarithmic

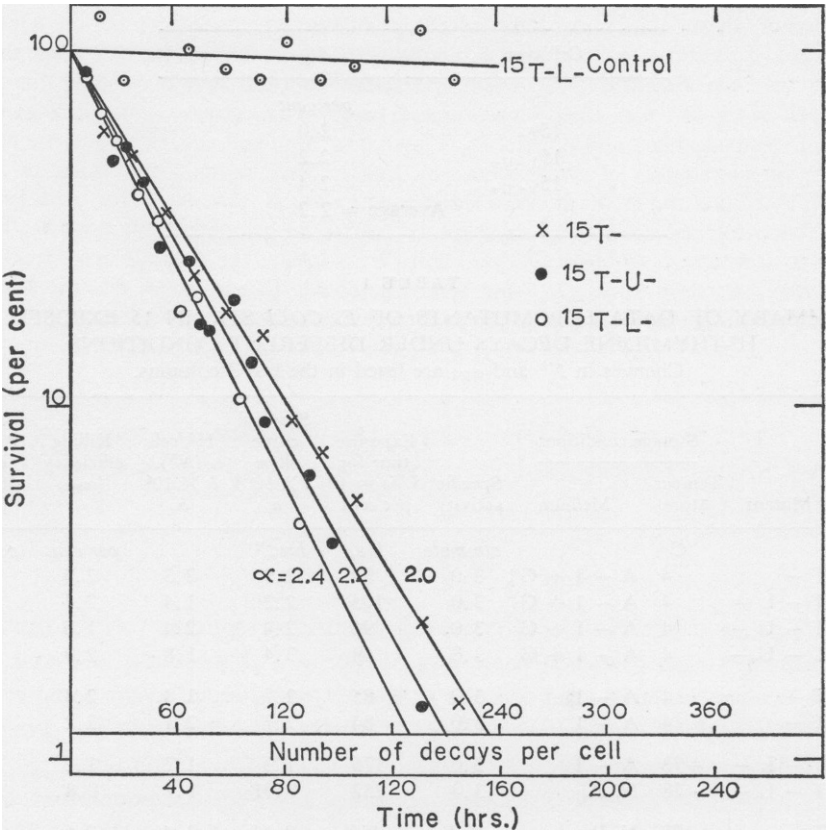

FIGURE 1 The survival of three mutants of *E. coli* strain 15; 15_{T-}, 15_{T-U-}, 15_{T-L-}, as a function of exposure time to H^3-thymidine decay and the number of labels/cell. The data for labeled cultures are corrected for loss of colony-forming ability in the unlabeled cultures. The survival curves are normalized to a single value of N^* (2.4×10^5 H^3-thymidines/cell) and so the differences in slope reflect different killing efficiencies (α_{H^3}), which are clearly small.

plot of the surviving fraction of bacteria (S/S_0) as a function of exposure time (t) for the three mutants 15_{T-}, 15_{T-U-}, 15_{T-L-}. After growth in media containing H^3-thymidine of specific activity 3.0 c/m mole the bacteria were stored at 4°C in A-1 medium, without supplements, but with glycerol at a final concentration of 10 per cent. Data for the 15_{T-L-} control culture are shown in Fig. 1. H^3-thymidine decay data in Fig. 1 are normalized for the loss of colony-forming ability occurring in the control cultures which amounts to 25 to 50 per cent over the total time of exposure. The curves in Fig. 1 are also normalized to a value of $N^* = 2.4 \times 10^5$ H^3/cell, which is the value determined experimentally for 15_{T-}. Therefore, significant differences in slope exhibited by the three mutants would reflect a different α_{H^3}. Values of N^* for the other mutants will be listed in Table 1. The killing efficiencies calculated from these data are:

Cell type	α_{H^3}
	per cent
15_{T-}	2.0
15_{T-U-}	2.2
15_{T-L-}	2.4
	Average = 2.2

TABLE I

SUMMARY OF DATA FOR MUTANTS OF *E. COLI* STRAIN 15 EXPOSED TO H^3-THYMIDINE DECAYS UNDER DIFFERENT CONDITIONS

Changes in N^* and α_{H^3} are listed in the last 3 columns.

Line No.	Mutant	Storage conditions: Temperature	Storage conditions: Medium	Specific activity	Exposure time for $S/S_0 = 10$ per cent	Survival curve slope $a \times 10^{-2}$ a	H^3/cell (N^*) $b \times 10^5$ b	Killing efficiency (α_{H^3})	Average killing efficiency (α_{H^3})
		°C		*c/mmole*	*hrs.*	*hrs.*$^{-1}$		*per cent*	*per cent*
1	T −	4	A − 1 + G‡	3.0	80	2.9	2.3	2.0	2.0
2	T − L −	4	A − 1 + G	3.0	105	2.2	1.4	2.4	2.4
3	T − U −	4	A − 1 + G	3.0	96	2.4	2.4	1.6	
4	T − U −	4	A − 1 + G	3.0	68	3.4	1.8	2.9	2.2
5	T −	4	A − 1§	3.0	85	2.7	1.8	2.4	2.3
6	T −	4	A − 1	3.0	84	2.7	2.1	2.2	
7	T − L −	−78	A − 1	3.0	176	1.3	1.7	1.2	
8	T − L −	−78	N. B.‖	3.0	252	0.91	1.7	0.8	
9	T −	−78	N. B.	0.9	456	0.51	1.4	0.6	0.67
10	T −	−78	N. B.	3.0	161	1.4	3.0	0.7	
11	T −	−78	N. B.	3.0	161	1.4	3.0	0.7	

‡ A − 1 + G = A − 1 plus glycerol at a final concentration of 10 per cent.
§ A − 1 = A − 1 minimal medium.
‖ N. B. = 0.8 per cent nutrient broth.

Thus, the average probability of producing death by a H^3 decay under these conditions is 0.022. The curves are definitely of the single hit type. Since the variation of α_{H^3} among the three cell types is probably within the limit of experimental errors we conclude that the α_{H^3} for each mutant is the same.

Summary of Data on Strain 15 Exposed to H^3-Thymidine Decay. Table I summarizes the variation in α_{H^3} for cultures exposed to H^3-thymidine decay under different conditions. The purpose of this table is to show the experimentally determined values of N^* as well as the calculated value of α_{H^3} for cultures exposed to these conditions. The value of N^* for the experiments shown in Fig. 1 is listed in the first part of Table I.

The labels/cell for the 10 experiments shown in Table I, which employ the same specific activity of H^3-thymidine, range from 1.4×10^5 to 3.0×10^5 thymidine.

Table I also shows the effect of removing glycerol from the storage medium. Glycerol was added to the medium with the thought that it might aid in stabilizing the control culture. The addition of glycerol does not significantly alter α_{H^3} (see lines 1, 5, and 6 in Table I). 15_{T-} cultures at 4°C in A-1 without glycerol give an $\alpha_{H^3} = 2.3$ per cent. This is to be compared with $\alpha_{H^3} = 2.0$ per cent for 15_{T-} in A-1 plus glycerol. Perhaps there is a small protection effect due to the presence of glycerol.

Nutrient broth on the other hand decreases α_{H^3} by about 50 per cent. That is, from Table I, lines 7 and 8, the ratio $(\alpha \text{ A-1}/\alpha \text{ N.B.})_{H^3} = 1.2/0.8 = 1.5$ for cultures of 15_{T-L-} stored at −78°C. Data from lines 9, 10, and 11 of Table I, for cultures stored in 0.8 per cent nutrient broth at −78°C, indicate that α_{H^3} is not dependent on the specific activity of H^3-thymidine and also that cultures stored in nutrient broth have a reduced killing efficiency.

The Temperature Dependence of the Killing Efficiency. The killing efficiency was measured at various storage temperatures and the data are shown in Fig. 2 where the efficiency, α_{H^3}, is plotted in per cent as a function of storage temperature in degrees Kelvin. A-1 storage medium was used in these experiments. The killing efficiency depends on temperature, and rises rather rapidly at the higher temperatures. Some of the variation is due, perhaps, to the change of phase from solid to liquid. If the curve for the liquid phase is extrapolated in a linear fashion to lower temperatures, $\alpha_{H^3} = 1.2$ per cent would be reached at a temperature of about −40°C. Thus, the curve is not linear between +4°C and −78°C. Most temperature effects, *e.g.*, radiation (Wood, 1954) and P^{32} decay (Stent and Fuerst, 1955), vary more slowly once the solid phase is reached and this is probably the case here.

Latent Killing Due to Decay. In the course of experiments at different storage temperatures we discovered what we term latent killing in cells that have been exposed to radioactive decay at −78°C. Latent killing is expressed when the frozen samples are thawed and stored in the liquid phase in A-1 medium that does not contain the necessary growth requirements.

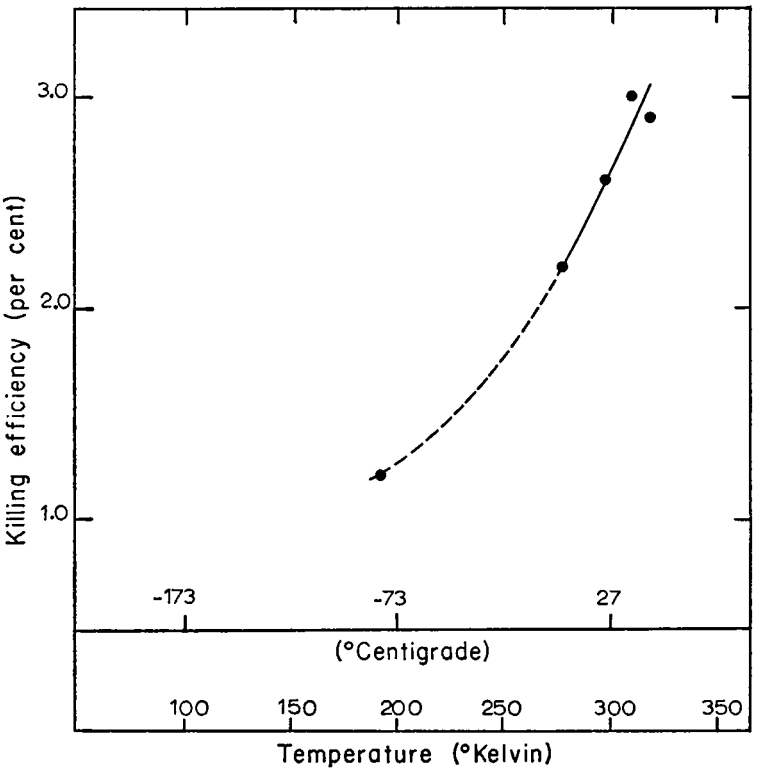

FIGURE 2 The variation in α_{H^3} with temperature of $15_{T\text{-}L}$ exposed to H^3 decay at the temperatures indicated. α_{H^3} depends on temperature and rises rather rapidly at the higher temperatures. A dashed line is used to represent the curve between the temperatures 195 to 277°K because there are no data in this interval and there is a solid-liquid transition in this interval.

An experiment indicating the rate and magnitude of the latent killing at 37°C is shown in Fig. 3. S/S_0 is plotted as a function of time after thawing for a control culture of $15_{T\text{-}L}$ and one that has been inactivated by radioactive decay to 1.5 per cent survival at −78°C. The cultures are thawed and then maintained at 37°C in A-1 medium. The total time elapsed in Fig. 3 is far less than the time in Fig. 1. Latent killing is not due to the number of decays that occur during these experiments, but rather to those that are accumulated during prior storage at −78°C.

In Fig. 3 we have plotted S/S_0 as 100 per cent at $t = 0$, the end of exposure at −78°C. The loss of colony-forming ability in the control culture is not appreciable during the time of the experiment. Latent killing is observed only if a labeled culture is allowed to accumulate decays at −78°C. If a culture is stored at 37°C immediately after labeling this fast killing component of the curve is not seen. Freezing and thawing *per se* is also without effect. The magnitude of latent killing is proportional

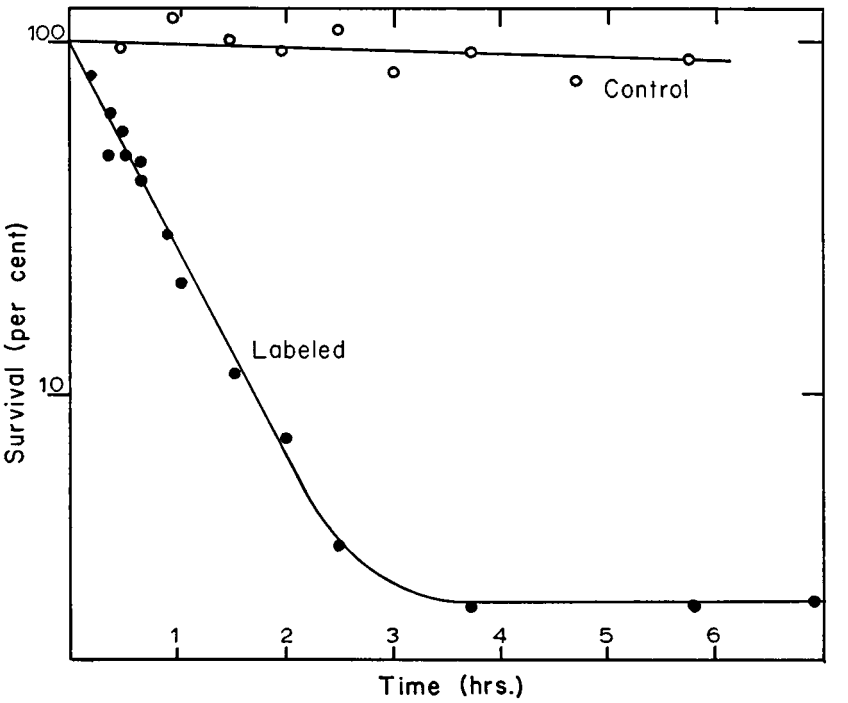

FIGURE 3 The time dependence of latent killing for 15_{T-L-} that have previously been exposed to H^3-thymidine decay at −78°C. Latent killing is very rapid and the number of decays that occur during this period of observation do not contribute to killing. The time scale is too short to show the actual nature of the final slope (compare with Fig. 1) which in fact is characteristic of α_{H^3} for this temperature. The magnitude of latent killing depends only on the previous history of exposure of the cells to radioactive decay at −78°C.

to the storage time at −78°C and is approximately a factor of 5 for each log of killing normally observed due to radioactive decay at −78°C. The time scale in Fig. 3 is too short to show the actual nature of the final slope which, in fact, is characteristic of the killing efficiency at that temperature. Thus, after latent killing has run its course the cells return to true radioactive decay killing at a rate that is characteristic of the storage temperature.

The Temperature Dependence of Latent Killing. The slopes for latent killing of 15_{T-L-} at 4° 25°, 37°, and 45°C, are listed in Table II, Typical survival curve slopes are also given for cultures labeled with the same specific activity of H^3-thymidine. The ratio of the latent killing to the H^3-thymidine decay slope is given in the last column. It is only the rate at which the latent killing occurs that is temperature-dependent. The magnitude of the effect as noted above depends only on the previous history of exposure to radioactive decay of the cells in the solid phase. Latent killing is extremely temperature-dependent and varies by a factor of 40 in the

TABLE II

TEMPERATURE DEPENDENCE OF LATENT KILLING FOR 15_{T-L-} IN A-1

The ratio latent killing decay slope, at the temperature indicated, is shown in the last column.

Temperature	Killing efficiency	H^3-thymidine decay slope	Latent killing slope	Latent killing slope / Decay slope
°C	*per cent*	*hrs.*$^{-1}$	*hrs.*$^{-1}$	
−78	1.2	−0.023	——	——
4	2.2	−0.042	−0.09	2
25	2.6	−0.50	−0.80	16
37	3.0	−0.058	−1.4	24
45	2.9	−0.056	−3.6	64

TABLE III

MUTATION PRODUCTION IN 15_{T-L-} INDUCED BY H^3-THYMIDINE DECAY AT −78°C

The labeled cultures have been inactivated to the per cent survival indicated in column 2. The mutation examined was lactose to non-lactose fermentation.

Exp. No.	Per cent Survival	Labeled		Unlabeled	
		No. of Mutants	Total No. of Colonies	No. of Mutants	Total No. of Colonies
M1	40	1	1,960	0	2,200
M2	8	1	3,876	0	10,166
M3	5	2	10,445	0	3,570
M4	3	2	4,140	0	2,420
M5	1.5	5	24,000	0	18,690
Total	——	11	44,421	0	37,046

temperature interval from 4° to 45°C. Finally it is observed that if one attempted to interpret latent killing as true suicide he would get an $\alpha_{H^3} = (64)\ (3.0) = 192$ per cent at 45°C.

Mutation Production by H^3 Decays. Data for mutation production induced by the H^3-thymidine decays in a labeled culture of 15_{T-L-} are shown in Table III. Viable cells were assayed for the production of the mutation lactose to non-lactose fermentation after inactivation at −78°C to the per cent survivals indicated. Mutants appear as colorless colonies when plated on EMB agar. Data are shown for the number of mutants detected among the total number of viable cells assayed in labeled and unlabeled cultures. In a culture subjected to H^3-thymidine decay 11 mutants were scored in 44,421 viable cells compared with 0 for a similar number of non-labeled cells. Both cultures were maintained under identical conditions, except for the presence of incorporated H^3-thymidine in the labeled culture. While we do not have sufficient data as of now to validate calculations of mutation frequencies it is evident that H^3-thymidine decays induce mutations in 15_{T-L-}.

DISCUSSION

The average α_{H^3} for three mutants of *E. coli* strain 15 is 2.2 per cent at 4°C and 1.2 per cent at −78°C. The killing efficiency for P^{32} is 2.0 per cent for B/r stored at −196°C (Fuerst and Stent, 1956). If we take α (−196°C)/α (−78°C)∼1 (Stent and Fuerst, 1955) then H^3-thymidine is 1.2/2.0 = 0.6 as effective as P^{32} in producing a lethality. If latent killing is included in the killing for H^3-thymidine–labeled bacteria stored at −78°C the killing efficiency would be increased to 1.8 per cent giving $\alpha H^3/\alpha P^{32} \sim 0.9$. Thus, at low temperatures it appears that the killing efficiency for H^3-thymidine inactivated bacteria is 0.6 to 0.9 of that previously found for P^{32} decay.

Cairns (1961) has published H^3-thymidine survival curves for T2 bacteriophage at 4°C and concludes that $\alpha H^3/\alpha P^{32} \sim 1.0$. Since Cairns was primarily interested is using H^3-thymidine as a label for autoradiography he did not determine N^* (per infectious unit) experimentally. Also it seems possible that his use of fluorodeoxyuridine to block thymidylate synthetase production might have increased the killing sensitivity. Also the ratio α(bacteria)/α(phage) may be different for H^3-thymidine decay than for P^{32} decay. With these facts in mind we conclude that our data for α_{H^3} are largely in agreement with those of Cairns.

However, our data are not in agreement with the recent work of Apelgot and Latarjet (1962) who measured the α_{H^3} for a B3/r bacterium at −196°C using H^3-thymidine and found $\alpha_{H^3} = 0.05$ per cent which is smaller than that reported here. Their work differs from that reported here in that their experimental conditions were different, a different bacterial strain was used, and a different method was used for the estimation of N^*.

It is appropriate to comment on the determination of N^* in our experiment since this is the most critical step in the calculation of α_{H^3}. The following reasoning indicates that the determination of N^* is consistent with the number of thymidine molecules per cell. Barner and Cohen (1954) have shown on the basis of limiting growth, that 1 μg of thymidine will support the growth of approximately 10^9 bacteria which gives 2.5×10^6 thymidine molecules/bacterium as an upper limit, which assumes that the bacterium can extract all the thymidine from the medium. For H^3-thymidine at a specific activity of 3.0 c/mmole we find 2.4×10^5 H^3-thymidine molecules/cell. At this specific activity there is on the average 1 labeled molecule in 8.9. Thus, we would calculate (2.4×10^5) $(8.9) = 2.1 \times 10^6$ thymidine molecules/bacterium. The agreement is satisfactory. Therefore, we conclude that the killing efficiencies as reported here for 15_{T-}, 15_{T-L-}, 15_{T-U-} labeled with H^3-thymidine are substantially correct. It would be quite interesting if the two related strains 15 and B gave such different killing efficiencies. This could indicate a completely different mechanism of killing in the two cases.

The dependence of the killing efficiency on temperature for H^3 decay at low temperatures, especially at the solid-liquid phase transition, differs from that for radia-

tion, and at higher temperatures in the liquid phase differs from that for P^{32} decay. The best estimate of $\alpha(4°C)_{H^3}/\alpha(-78°C)_{H^3} = 1.2$ as compared with 2 to 3 for radiation, and $\alpha(40°C)_{H^3}/\alpha(4°C)_{H^3} = 1.35$ as compared with 1.15 for P^{32} decay. There is apparently no sharp rise in α_{H^3} at the solid to liquid phase transition of the kind that is characteristic of radiation inactivation. The work of Wood (1954) and Stapleton and Edington (1956) indicate an increase in radiation sensitivity of microorganisms by a factor of 2 to 3 mainly due to the phase transition. Stent and Fuerst (1955) report $\alpha(40°C)_{P^{32}}/\alpha(4°C)_{P^{32}} = 1.15$ for P^{32} decay in bacteriophage. The low recoil energy of the He^3 nucleus, average about 1 ev, means that statistically more decays from H^3 might be affected by thermal energy than for P^{32}, where the average S^{32} recoil energy is 30 ev.

The mechanism responsible for latent killing is not understood. However, the reduced killing efficiency for labeled cells stored in 0.8 per cent nutrient broth at −78°C and preliminary experiments indicating that resuspension of samples in nutrient broth partially or completely inhibits the latent killing suggests that long-lived radicals are involved. Latent killing is being investigated further. This effect has not been reported for P^{32} decay.

While the small number of mutants observed prevents an accurate, calculation of mutation frequency, especially as a function of time exposed to H^3-thymidine decays, it is clear that mutations are produced in abundance. The mutation frequency is very approximately 1 in 5,000 viable cells for a culture inactivated to 1.5 per cent survival (smallest survival value used in mutation experiments) by exposure to decay at −78°C. For a radiation dose of 10,000 r a typical value for mutation frequency for a particular reversion would be 5 in 10^7 viable cells, (Demerec and Sams, 1958; McDonald and Wyss, 1959; Deering, 1962) which gives a maximum survival of 50 per cent for a B/r bacterium irradiated under similar conditions. For single hit kinetics, for one particular mutation,

$$M/M_0 = 1 - \exp(-kD)$$

where M = number of mutants observed for a dose D
M_0 = number of viable cells remaining after a dose D
D = exposure dose in units of mean lethal dose (MLD)
k = constant related to rate at which mutants are produced in units of $(\text{MLD})^{-1}$

For small values of e^{-kD}, M/M_0 is very nearly equal to kD. For H^3-thymidine decay $M/M_0 = 1/5000$ and 1.5 per cent survival corresponds to a MLD = 4.2 leading to $k_{H^3} = 4.8 \times 10^{-5}$/MLD. The same process when applied to the above data for radiation leads to a k for radiation of 7×10^{-7}/MLD which is much smaller (70-fold) than that just calculated for H^3-thymidine decay. Although this calculation is for different genetic groups in different bacterial strains it strongly suggests that mutation production from H^3-thymidine decays is due to chemical changes asso-

ciated with the transmutation process and not due to radiation damage due to the β-particle accompanying the transmutation process.

For P^{32} decay about 1 in every 5×10^4 disintegrations results in the production of an auxotrophic mutant of the type recognizable by replica-plating broth agar–surviving colonies on minimal agar (Kaudewitz, Vielmetter, and Friedrich-Freska, 1958). For H^3-thymidine approximately 1 decay in 10^6 results in the production of the specific mutation lactose to non-lactose fermentation. It would seem that mutations are produced by H^3-thymidine decay at a comparable rate since the assay employed in the case of P^{32} decay must have detected at least the order of 20 different kinds of mutants.

Although the mechanism by which H^3 decays cause mutations and bacterial killing is not understood, the data presented here indicate that these phenomena are brought about by chemical changes in the transmutation process as opposed to β-particle radiation damage.

This manuscript was written after one of us (S.P.) moved to The Pennsylvania State University. It is a pleasure to acknowledge assistance from Professor Ernest Pollard in the preparation of the manuscript. His suggestions, criticisms and genuine interest in this work are sincerely appreciated. Several helpful discussions with Professor William Ginoza are also acknowledged.

This research was supported by the United States Atomic Energy Commission under Contract AT-04-1-Gen-12.

Received for publication, June 2, 1962.

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