DIFFERENTIAL MUTATION PRODUCTION BY THE DECAY OF INCORPORATED TRITIUM COMPOUNDS IN *E. COLI*

S. PERSON *and* R. C. BOCKRATH, JR.
From the Biophysics Department, Walker Laboratory, The Pennsylvania State University, University Park

ABSTRACT We have studied the differential mutation production by the decay of incorporated tritium compounds in *E. coli* (WWU) using DNA-seeking precursors (H^3-thymidine), RNA-seeking precursors (H^3-uracil, H^3-uridine), and protein-seeking precursors (H^3-histidine, H^3-proline). In particular we have determined the reversion frequency of an arginine locus. The reversion frequency is measured in units of revertants/surviving bacteria/H^3 decay, and has an average value of 1.84×10^{-8} for H^3-uridine and H^3-uracil, 0.67×10^{-8} for H^3-thymidine, and 0.28×10^{-8} for H^3-proline and H^3-histidine. Thus, the revertants are produced most effectively by H^3 decays when the label is introduced in the form of an RNA precursor. The macromolecular distribution of the label shows that 5 to 8 per cent of the H^3-uridine or H^3-uracil is incorporated into DNA.

INTRODUCTION

The decay of incorporated H^3- thymidine in mutants of *E. coli* strain 15 has been shown to have a killing effect comparable to that for P^{32} decay (Person and Lewis, 1962). Comparative killing efficiencies using the decay of incorporated H^3-thymidine, H^3-uridine, and H^3-histidine (Person, 1963) and H^3-thymidine, H^3-uridine, and H^3-leucine (Rachmeler and Pardee, 1963) show the decays from DNA precursor (H^3-thymidine) to be more effective than those from RNA precursor (H^3-uridine), which in turn are more effective than those from protein precursors (H^3-histidine, H^3-leucine). The same order of effectiveness was observed by Rachmeler and Pardee (1963) for the loss of enzyme forming ability (β-galactosidase) in *E. coli*. Thus, the decay of H^3-thymidine from within the DNA is most effective in destroying these biological activities.

Preliminary mutation studies indicated H^3-thymidine decay (Person and Lewis, 1962) to be nearly as mutagenic as P^{32} decay (Kaudewitz, Vielmetter, and Friedrich-Freska, 1958). We have extended the use of heavy differential tritium labeling

to an examination of mutagenesis produced by decays from different H^3 compounds.

In experiments reported here we have studied the reversion of an arginine locus in *E. coli*, that is the conversion of mutant bacteria from an arginine dependent to an arginine independent state. The reversion frequencies were determined using H^3 decays from H^3-thymidine, H^3-uridine, H^3-uracil, H^3-proline, and H^3-histidine. Unexpectedly, decays from incorporated H^3-uridine (and H^3-uracil) have been found 3 times more effective than those from H^3-thymidine, and 7 times more effective than those from either H^3-proline or H^3-histidine.

MATERIALS AND METHODS

A mutant of *E. coli* strain 15, referred to here as WWU, which is deficient in thymidine, uracil, tryptophan, methionine, proline, and arginine was used in these experiments. We will refer to this stock as the mutant strain and the mutations from arginine dependence to independence produced by H^3 decays as revertants. The mutant strain was obtained from Wax[1] who added a uracil requirement to the organism 1024-4/6 that he obtained from Weatherwax.[2]

The growth requirements were supplied by 2 μg thymidine, 30 μg uridine, and 50 μg L-tryptophan, L-methionine, L-proline, and L-arginine per milliliter A-1 solution. A-1 solution consisted of 2 gm NH_4Cl, 6 gm Na_2HPO_4 (anhydrous), 3 gm KH_2PO_4, 5 gm NaCl, 0.115 gm Na_2SO_4, 0.34 gm $MgCl_2 \cdot 6H_2O$ and 4 gm glucose per liter of distilled water.

The labeling procedure and the procedure for determining the number of H^3 decays/hour/bacterium have been stated previously (Person and Lewis, 1962). Briefly, they are as follows: A dilution of a log phase culture growing in supplemented A-1 at 37°C with aeration was added to the desired quantity of H^3 compound[3] in similar growth medium

---

[1] Richard Wax, Biophysics Department, The Pennsylvania State University, University Park, Pennsylvania.

[2] Robert Weatherwax, Biology Department, New Mexico Highlands University, Los Vegas, New Mexico.

[3] H^3-thymidine, H^3-uridine, H^3-proline, and H^3-histidine were obtained from Schwarz Bioresearch, Orangeburg, New York. H^3-thymidine and H^3-uracil were obtained from New England Nuclear Corp., Boston, Massachusetts. H^3-thymidine was supplied at 3 and 6.6 c/mmole, respectively. H^3-uridine, H^3-uracil, H^3-proline, and H^3-histidine were supplied at approximately 1 c/mmole. All H^3 compounds were obtained at an activity of 1 mc/ml. Isotopes were used at the following specific activities (S. A.) and concentrations:

H^3-compound	Final S. A.	Final concentrations
	c/mmole	*μg/ml*
H^3-thymidine	3–6.6	2
H^3-uracil	0.1–0.2	15
H^3-uridine	0.25–0.5	30
H^3-proline	1	3–5
H^3-histidine	1	3–5

and conditions. Cell counts during growth were determined with a Coulter counter[4] and showed that growth was logarithmic throughout the labeling period. After 5 to 6 cell divisions (final titer 6×10^8/ml) in highly radioactive medium, the cells were filtered and resuspended in A-1 medium and stored at 4°C to accumulate decays. The rate of accumulation of decays, in units of decays/hour/bacterium, was determined by cell counts[4] and radioactivity determinations[5] on aliquots of the stored bacterial resuspensions. Correction for self-absorption of 60 per cent was used, as indicated by chemical extraction and hot TCA (trichloroacetic acid) dissolution of the nucleic acid[6] from within the bacterium. The self-absorption was the same in cultures labeled with different H^3 compounds.

Dilutions of stored cultures were made in A-1 solution at various times and plated for viability on A-1 titer plates hardened with agar at a concentration of 1.5 per cent. These titer plates were supplemented with tryptophan, methionine, proline, and arginine at 100 μg/ml, and uracil and thymidine at 20 μg/ml. Platings for arginine revertants were done at the same time by plating aliquots of the stored cultures directly on search plates. These plates were similar to titer plates but contained no arginine. In addition, they were fortified with nutrient broth at 0.2 gm/liter. In the experiments quoted in Fig. 4 variable amounts of nutrient broth fortification were used as indicated. Viability and revertant plates were incubated for 25 hours at 37°C to produce distinct colonies. The number of colonies was used as a measure of the viability and mutability.

In experiments to determine the distribution of radioactivity, bacteria labeled with H^3-thymidine, H^3-uridine, and H^3-uracil were extracted with TCA and NaOH to separate their nucleic acids. This procedure, due to Schmidt and Thannhauser (1945) and Schneider (1945), involved 0.3 M TCA extraction at 4°C (acid-soluble fraction), overnight incubation at 37°C in 1 N NaOH (RNA rendered soluble), heating in boiling water bath for 30 minutes in 0.3 M TCA (DNA rendered soluble), and the dissolution of the remaining residue in 1 N NaOH. This method of fractionation of the cells is not absolute, and probably there is a small contamination of each fraction with the others.

Radioactivity in all fractions was determined by liquid scintillation counting. In all cases 0.1 ml of sample was dispersed in 20 ml of liquid scintillation fluid. Labeled cell suspensions and the chemical reagents employed in the Schmidt-Thannhauser-Schneider procedure produced the same quenching effect (to within 5 per cent).

RESULTS

The Kinetics of Revertant Production and Loss of Viability. Both loss of viability and revertant production are observed in the radioactive cultures. Results from an H^3-uridine–labeled culture are shown in Fig. 1. The upper curve in Fig. 1 shows production and survival of arginine revertant bacteria. The number of surviving revertants is plotted as a function of the number of decays/bacterium.

The revertants/milliliter shown in the figure are the net number. That is, the

[4] Coulter cell counter, Coulter Electronics, Hialeah, Florida.

[5] Tri-carb liquid scintillation spectrometer, Packard Instrument Co., La Grange, Illinois.

[6] Chemical extraction by the Schmidt-Thannhauser-Schneider procedure or dissolution of whole cells in 10 per cent TCA at 95°C for 20 minutes serves equally well to solubilize incorporated label. The use of these methods in determining self-absorption will be discussed in a future publication.

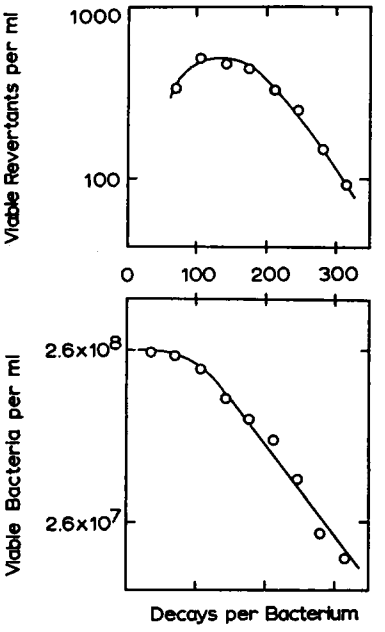

FIGURE 1 The upper curve in Fig. 1 is a result of the production and survival of arginine revertant bacteria. The number of surviving revertants is plotted as a function of the number of decays/bacterium. Cultures were labeled with H^3-uridine, stored to accumulate decays, and plated at various times. The revertant curve initially shows an increase in the number of revertants, followed by flattening, and finally a decrease in number, as the number of H^3 decays/bacterium increases. The lower curve shows the surviving bacteria on a semilogarithmic scale as a function of the number of decays/bacterium. The number of surviving revertants/surviving bacteria is the ratio of the revertant curve to the viability curve at any number of decays/bacterium.

number of spontaneous revertants obtained by plating an equal volume of a control culture has been subtracted. The number of revertants on a control Petri dish is approximately 7 regardless of the size of the inoculum used (10^6 to 10^8 viable bacteria were plated). These revertants arise mainly from growth on the plate in the presence of 2.5 per cent nutrient broth (final yield of about 10^{10} bacteria per plate). The number of revertants in the liquid culture is small, less than 1 in 10^8. Control cultures plated for both reversion and viability showed little variation over the course of the experiments.

The number of accumulated decays/bacterium was calculated from the rate of decay/bacterium/hour and the time (hours) between filtering and plating. The rate of decay in all experiments for all H^3 compounds used was of the order of 3 decays/bacterium/hour.

The number of revertants/milliliter produced by H^3 decays initially shows an absolute increase in number, followed by flattening, and finally a decrease, as the number of accumulated decays/bacterium increases.

The survival curve for WWU shown in the lower part of Fig. 1 is of the multihit type. In different experiments there is some variation in the extrapolation number.

The Reversion Frequencies in H^3-Thymidine– , H^3-Uridine– , H^3-Uracil– , H^3-Proline– , and H^3-Histidine–Labeled Cultures. A reversion frequency can be obtained from the data as presented in Fig. 1. Since Fig. 1 shows the number of surviving revertants/milliliter and the number of surviving bacteria/milliliter, we

can determine the number of surviving revertants/surviving bacteria at a certain number of decays/bacterium by taking the ratio of

$$\frac{\text{Surviving revertants/milliliter}}{\text{Surviving bacteria/milliliter}}$$

Each point in Fig. 2 is determined in this way. We have plotted this number as a function of accumulated decays/bacterium on a linear scale for decays from H^3-thymidine, H^3-uridine, and H^3-proline. The frequencies of reversion in the arginine locus by H^3 decay are the slopes of the lines in Fig. 2. Their units are surviving revertants/surviving bacteria/H^3 decay and are designated hereafter as k values. Thus, our reversion frequencies are expressed on a per H^3 decay basis, which serves as a basis for comparing frequencies by different H^3-labeled compounds.

The data shown in Fig. 2 are from two experiments. It is clear that H^3-uridine decays produce revertants at a substantially higher rate than either those from H^3-thymidine or H^3-proline. The reversion frequencies are $k_{(H^3\text{-uridine})} = 2.1 \times 10^{-8}$, $k_{(H^3\text{-thymidine})} = 0.78 \times 10^{-8}$, and $k_{(H^3\text{-proline})} = 0.30 \times 10^{-8}$, which are in the ratio of 7 : 2.6 : 1, respectively. Thus, on a per decay basis, H^3-uridine produces revertants at a rate 2.7 times that of H^3-thymidine and 7.0 times that of H^3-proline. While the actual k values change somewhat from experiment to experiment, the ratios are always in this order and nearly of the magnitude stated.

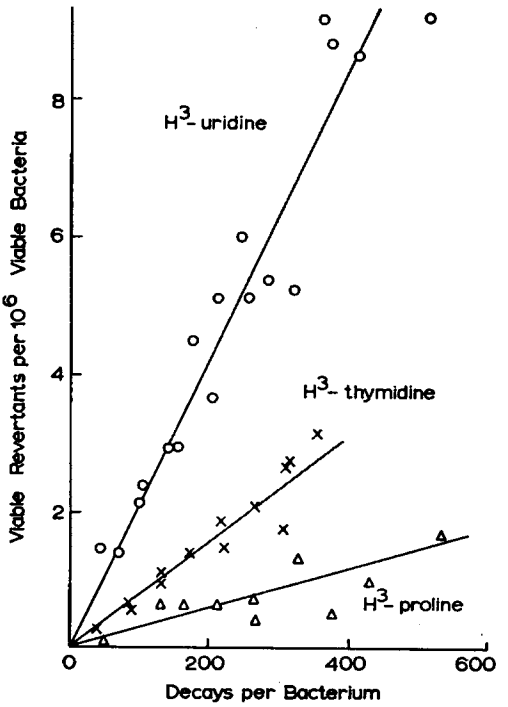

FIGURE 2 Fig. 2 is a linear graph of the number of surviving revertants/surviving bacteria as a function of the number of decays/bacterium. Each point is determined from data on surviving revertant and viable bacteria mentioned in the legend of Fig. 1. The reversion frequencies, revertants/surviving bacteria/H^3 decay, are the slopes of these lines. Data are shown for H^3-uridine, H^3-thymidine, and H^3-proline. It is clear that H^3-uridine decays revert the arginine locus of WWU at a significantly greater rate than either those from H^3-thymidine or H^3-proline.

Data of the same nature are shown in Fig. 3 for decays from H^3-uracil and H^3-histidine. The data for H^3-uracil decays are from five experiments and those for H^3-histidine are from four experiments. The reversion frequency for H^3-uracil is 1.72×10^{-8} and for H^3-histidine is 0.30×10^{-8}. Hence, H^3-uridine and H^3-uracil have similar k values, as do H^3-proline and H^3-histidine.

We have listed in Table I the reversion frequencies (k), the killing efficiencies (α), and the rate of decay/bacterium (λN^*) for all our experiments including those shown in Figs. 2 and 3.

The Cellular Distribution of H^3-Labeled Compounds. Table II shows the average cellular distribution of H^3-thymidine, H^3-uridine, and H^3-uracil for several of the experiments listed in Table I. For H^3-uracil approximately 80 per cent of the total and 90 per cent of the total incorporated activity (the total activity minus acid-soluble fraction) resides in the RNA fraction. For H^3-uridine the percentages are not significantly different. H^3-labeled thymidine has approximately 80 per cent of the total activity and 90 per cent of the total incorporated activity in the DNA fraction. We have not examined the distribution of protein presursor labels. However, for the related mutant 15_{T-L-}, using enzymatic digestion, we have shown that a similar high percentage of amino acid label is incorporated into protein (Person, 1963). Finally we note that the incorporation of label from H^3-uracil or H^3-uridine into DNA is 5 to 8 per cent.

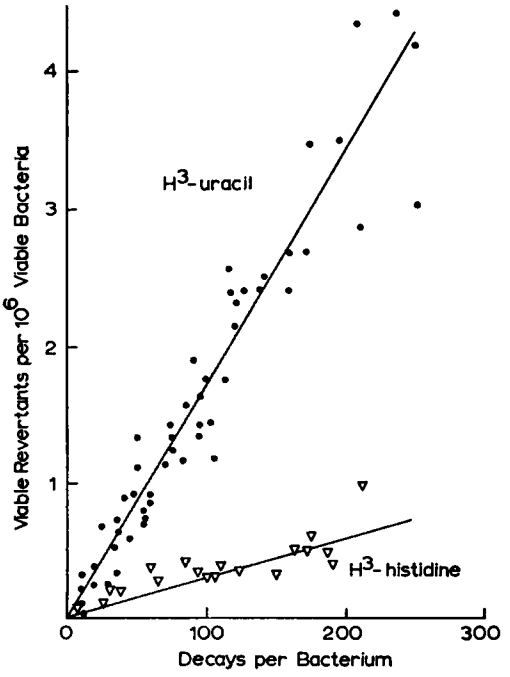

FIGURE 3 Fig. 3 shows data from which the reversion frequencies for H^3-uracil and H^3-histidine were determined. The reversion frequency for H^3-uracil decays is very similar to that for H^3-uridine and the reversion frequency for H^3-histidine is very similar to that for H^3-proline.

TABLE I

CONSTANTS DETERMINED FROM H^3 DECAY

Date	RNA precursor (*a* uracil) (*b* uridine) ‡λN^*	§k(× 10^8)	‖α	DNA precursor (thymidine) λN^*	k(× 10^8)	α	Protein precursor (*c* proline) (*d* histidine) λN^*	k(× 10^8)	α
			per cent			*per cent*			*per cent*
Aug. 19, 1962	*b* 2.95	2.1	1.13	3.70	0.78	1.05	*c* 5.48	0.30	0.78
Sept. 27	*b* 4.35	2.1	1.28	3.70	0.78	1.32	*c* 5.55	0.30	0.48
Mar. 4, 1963	*a* 0.88	–	0.96	–	–	–	–	–	–
	a 1.10	–	0.90	–	–	–	–	–	–
	a 3.90	–	1.00	–	–	–	–	–	–
Mar. 24	*a* 2.02	1.9	1.3	–	–	–	–	–	–
June 26	*b* 4.33	2.0	0.87	3.45	0.35	0.89	–	–	–
July 3	*a* 1.61	2.0	1.7	1.75	1.08	1.25	–	–	–
July 10	*a* 2.28	1.6	1.0	2.84	–	1.70	*d* 2.4	0.35	0.95
July 12	*b* 5.05	1.94	1.37	2.90	0.38	1.82	–	–	–
July 16	*a* 1.77	1.8	1.6	–	–	–	–	–	–
July 19	*a* 4.05	1.3	1.2	–	–	–	–	–	–
Aug. 8	*b* 5.70	1.74	0.45	–	–	–	–	–	–
Aug. 19	–	–	–	–	–	–	*d* 4.5	0.28	1.10
Aug. 20	*b* 6.60	–	0.60	–	–	–	*d* 7.30	–	0.68
Aug. 22	*b* 7.30	1.82	0.47	–	–	–	*d* 6.80	0.15	0.51
Aug. 26	–	–	–	–	–	–	*d* 3.5	0.28	0.93
Average	*a*	1.72	1.21		0.67	1.34		*c* 0.30	*c* 0.63
	b	1.95	0.88					*d* 0.26	*d* 0.83
Precursor average	1.84 ± 0.26			0.67 ± .28			0.28 ± 0.07		

In this table we have listed the H^3 compounds used, the reversion frequency (k), and the killing efficiency (α) for the experiments indicated. The rate of decay/bacterium is also shown. The average k and α values are listed at the bottom of the table. The error limits shown at the bottom of the table refer to one standard deviation.

‡ The units of λN^* are decays/bacterium/hour.

§ k is the probability that a single H^3 decay produces a revertant in a surviving bacterium and is expressed as revertants/surviving bacteria/H^3 decay.

‖ α is the probability that a single H^3 decay produces lethality and is expressed as this percentage.

TABLE II

THE CELLULAR DISTRIBUTION OF H^3-URIDINE, H^3-URACIL, AND H^3-THYMIDINE IN WWU

Fraction	H^3-uracil	H^3-uridine	H^3-thymidine
Acid-soluble	11.5	11.1	11.8
RNA	79.5	82.6	8.0
DNA	7.9	5.0	78.5
Residue	0.9	1.6	1.7

The Schmidt-Thannhauser-Schneider method was used to separate the nucleic acids. The number given for each fraction is the average per cent of the total activity in that fraction as determined from several experiments.

The Effect of Nutrient Broth Fortification. It has been shown for revertants produced by ultraviolet light that a small amount of nutrient broth added to the revertant Petri dish aids revertant expression (Witkin, 1956), so that more revertants are observed than if plating had been on minimal medium. This procedure has become widely used in research on revertant production by ultraviolet light. Therefore, we varied the concentration of nutrient broth to see if a similar effect existed for revertants produced by H^3 decays. Our results are shown in Fig. 4,

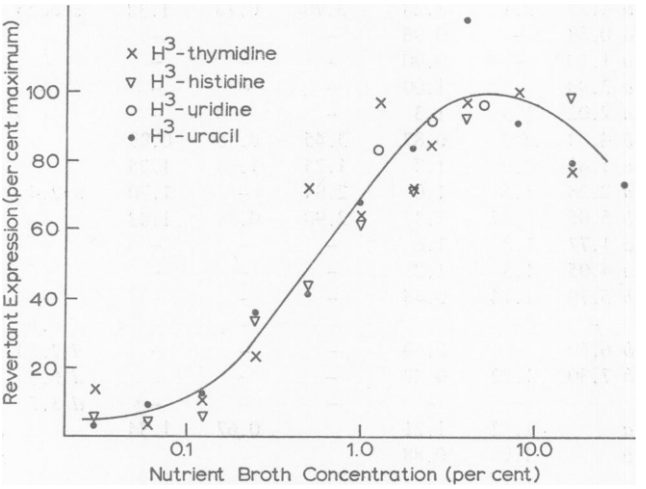

FIGURE 4 The effect of varying the amount of nutrient broth/plate. Cultures labeled with either H^3-uracil, H^3-uridine, H^3-thymidine, or H^3-histidine were stored in A-1 at 4°C until they had accumulated 100 to 150 decays/bacterium. Aliquots of the cultures were then plated at the nutrient broth concentrations indicated, where 100 per cent refers to a concentration of 8 gm/liter. The number of revertants from control cultures has been subtracted from the data shown above. Maximum expression was determined from an average of two or three of the largest numbers obtained. The revertant expression as a function of nutrient broth concentration is similar for all the H^3-compounds used.

where we have plotted the per cent of maximum number of revertants as a function of nutrient broth concentration. One hundred per cent nutrient broth fortification refers to nutrient broth at a concentration of 8 gm/liter. Radioactive cultures of WWU were stored at 4°C until they had accumulated 100 to 150 decays/bacterium. At this time aliquots were plated on search plates with nutrient broth fortification varied from 0.03 to 32 per cent. Data are shown for revertants produced by H^3-thymidine, H^3-uracil, H^3-uridine, and H^3-histidine decays. At very low broth concentrations only a small percentage of the maximum number of revertants is expressed. As the nutrient broth concentration is increased, the number of revertants expressed per Petri dish increases until a maximum number is reached at a nutrient

broth concentration of approximately 2.5 per cent. At concentrations greater than 10 per cent, the number of revertants actually shows a decrease with increasing broth concentration. The response to varying concentrations of nutrient broth appears to be independent of the H^3 compound used to produce the revertants. For this reason, and that maximum expression is reached near a nutrient broth fortification of 2.5 per cent, we have used this amount of nutrient broth fortification in all search plates. Witkin (1956) has indicated that this amount of nutrient broth permits a rate of protein synthesis which gives maximum expression of revertants produced by ultraviolet light. It remains to be seen if the decrease in revertant expression in the region of high nutrient broth fortification is of any inherent interest.

DISCUSSION

The kinetics of reversion as a function of accumulated decays/bacterium are similar to those for mutation production found in bacteriophage (Tessman, 1959) and tobacco mosaic virus (TMV) (Gierer and Mundry, 1958) by the continued presence of chemical mutagens. Initially decays produce revertants in bacteria that have not accumulated a lethal decay and so the number of revertants/milliliter increases. However, as the decays/bacterium accumulate the probability of survival of a H^3 decay produced revertant decreases, and ultimately the number of revertants/milliliter decreases.

It is clear from reversion frequencies displayed in Figs. 2 and 3, and the slope constants stated in Table I, that the decays from incorporated H^3-uracil or H^3-uridine produce revertants at a higher rate than decays from incorporated H^3-thymidine, H^3-histidine, or H^3-proline. The effectiveness of H^3-thymidine is between that of the labeled amino acids and H^3-uridine or H^3-uracil.

Since H^3-uracil and H^3-uridine are essentially equivalent RNA precursors, we have averaged their reversion frequencies at the bottom of Table I. Since the two amino acid labels are protein precursors we have averaged their reversion frequencies. Therefore, the average reversion frequencies for decays from H^3-labeled compounds that are precursors of RNA, DNA, and protein are 1.84×10^{-8}, 0.67×10^{-8}, and 0.28×10^{-8}, respectively, and are in the ratio of 6.7 : 2.4 : 1.0. The ratio of RNA : DNA is 2.7 : 1.0.

The killing efficiencies are of interest in that the lethal effectiveness of the precursors used is in the decreasing order DNA : RNA : protein. While the actual killing efficiencies and their ratios are not identical with those reported for *E. coli* $15_{T\text{-}L}$ (Person, 1963), they are similar and in the same order. Thus, H^3 decays from incorporated H^3-thymidine are most effective in causing lethality while those from H^3-uridine (or H^3-uracil) are most effective in producing reversion of the arginine locus.

We have considered two interpretations of our data with regard to the mecha-

nism of H^3 decay damage: (*a*) Tritium decay damage, both lethal and mutagenic, is entirely the result of β-particle radiation damage. Then, the lack of parallel between the reversion frequencies and the killing efficiencies requires that the vital centers for viability and arginine reversion have different locations in the bacterium. (*b*) Tritium decay damage, both lethal and mutagenic, is mediated in part by a local phenomenon at the site of the nuclide, and in part by β-particle radiation damage. It seems to us that the distribution of protein and RNA is similar in bacteria (Caro, 1962; Caro and Forro, 1962). Hence, the striking difference in the k values for RNA and protein would be interpreted as evidence favoring a local phenomenon. In the case of a local phenomenon, then the actual values for reversion frequencies would be expected to be different and would reflect the importance of the various labeled molecular species to the cell function examined.

The data of Table II indicate that the incorporation of label from an RNA precursor label into DNA is of the order of 5 to 8 per cent. In another mutant of this strain we have reported an incorporation of 8 per cent (Person, 1963). Caro and Forro (1962) in still another mutant report 8 per cent. Since uridine will not fill the thymidine requirement, the incorporation of RNA precursor into DNA very likely occurs as a conversion of uracil or uridine to deoxycytosine or deoxycytidine. Chromatographic recovery of label in the position of cytosine from hydrolyzates of H^3-uridine labeled cultures offers support for this conversion.

The molecular species receiving H^3-uridine or H^3-uracil has been shown to be RNA and DNA. Thus, the existence of a local effect and the unexpectedly high reversion of the arginine locus by incorporated H^3-uridine or H^3-uracil could refer to an RNA species intimately related to the replication of genetic information or to a cytosine "hot spot" in DNA.

To suggest an actual mechanism by which an H^3 decay leads to an expressed revertant requires further understanding of the relation between the primary alteration caused by the H^3 decay and the final expression of this alteration in the produced revertant. Experiments are in progress to determine the nature of this relation.

This research was supported by grants from the United States Public Health Service and by the National Aeronautics and Space Agency. It is always a pleasure to acknowledge discussions with Professor Ernest Pollard. In addition, we acknowledge discussion of our data with Mary Osborn, Brent Benson, Marshal Edgell, Frederick Funk, and Richard Wax. We sincerely appreciate the technical assistance of Miss Cora Jean Schaunberger.

Received for publication, October 15, 1963.

REFERENCES

CARO, L. G., *J. Biophysic. and Biochem. Cytol.*, 1962, **9**, 539.
CARO, L. G., and FORRO, F., JR., *J. Biophysic. and Biochem. Cytol.*, 1962, **9**, 555.
GIERER, A., and MUNDRY, K., *Nature*, 1958, **182**, 1457.
KAUDEWITZ, F., VIELMETTER, W., and FRIEDRICH-FRESKA, H., *Z. Naturforsch.*, 1958, **13**, 793.

PERSON, S., *Biophysic. J.*, 1963, **3**, 183.
PERSON, S., and LEWIS, H. L., *Biophysic. J.*, 1962, **2**, 451.
RACHMELER, M., and PARDEE, A. B., *Biochem. et Biophysic. Acta*, 1963, **69**, 62.
SCHMIDT, G., and THANNHAUSER, S. J., *J. Biol. Chem.*, 1945, **161**, 83.
SCHNEIDER, W. C., *J. Biol. Chem.*, 1945, **161**, 161.
TESSMAN, I., *Virology*, 1959, **9**, 375.
WITKIN, E., *Cold Spring Harbor Symp. Quant. Biol.*, 1956, **21**, 123.