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Progress Report

NASA Contract NsG324

This Covers the Period Until
July 1, 1964

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The activity on this contract has been considerable in this period of time. A very important piece of work has been completed on the effect of ionizing radiation on genetic transcription. A copy of an account of this which has been submitted as a letter to Science is included as Appendix 1. Another completed study involves the rate of mutation in cells grown in D_2O. This has disposed of the suggestion that the process of mutation consists of a"tunneling"of a hydrogen nucleus from one base to another in DNA. Such a suggestion cannot be advanced as a major explanation of mutations. A short summary is included as Appendix 2.

Work has been going forward on the centrifugation of cells of E. coli. It has been shown that cells which are exposed to as low as 100g actually have a modification in their behavior. We have looked at this from the point of view of thymine uptake, which would be concerned with the formation of DNA, and also from the point of view of the induction of an enzyme, which would correspond to the transcription of the DNA. Preliminary experiments in the latter case indicate considerable effect of centrifugation. The thymine uptake is affected, but not nearly as much.

A very successful workshop on the "Effect of Ionizing Radiation on Bacteria" was conducted and a preliminary transcript of this will be sent out within two weeks. It is hoped to have a corrected copy so that it can be made available to the NASA office, and they may elect to publish it or not as they please. There will be some difficulty regarding the diagrams in the preliminary transcript but we do not anticipate much trouble in this direction for the final form.

A very great effort has been going forward with the aim, an understanding of radiation effects on bacteria. This has involved studies of thymine labeling.

uracil uptake, induction of enzymes, as well as a projected set of three experiments involving the physical nature of the DNA. It looks as though we are very close to an understanding of what ionizing radiation does to simple cells.

Progress has been made with the high intrinsic viscosity machine. This is now working and the viscosity preparations of DNA has been measured. It is hoped to use this to study the variation in viscosity in DNA as a function of the cell cycle. Work on this has been pushed quite hard.

The design of a spectrophotometric method of studying single cells has begun. We are hoping to suggest this as a method of looking for biological molecules in samples of soil on the surface of Mars. This is part of the exobiology effort that we are putting in.

Work on the mutagenic nature of tritium decay has been advancing rapidly. This work bears on the rather mysterious process of "stabilizing" mutants. The draft of a paper in this area is included as Appendix 3.

The grant has been of enormous use, and is enabling, a very vigorous push to take place this summer.

An additional faculty member: Dr. Wallace E. Snipes will be paid partly on the grant. He is going to study electron spin resonance effects, partly in association with radiation action and partly as a general tool for studying living things.

THE EFFECT OF IONIZING RADIATION ON GENETIC TRANSCRIPTION

ABSTRACT

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Cells of E. coli grown on maltose can be induced to produce betagalactosidase by the addition of thiomethyl galactoside (TMG). If cells are irradiated shortly after induction, the transcription of the DNA ceases and the enzyme produced by the messenger RNA is observed to reach a maximum. This enables the calculation of the half-life of unstable messenger RNA. The half-life for this decay is readily measurable, and values are given over a temperature range from 17°C (5.2 min.) to 45°C (0.56 min.). These agree very well with half-lives measured by others by inducing for short times and watching the course of enzyme formation. The rate of transcription is involved in the kinetics of cessation and values for the rate of transcription can be measured. Arrhenius plots for this rate and the rate of decay are given and the activation energies measured are in the range of 16,000 cal/mole. The cessation of transcription is linked to the degradation, possibly of only one strand, of DNA.

Author

It has been suggested that one important action of ionizing radiation is concerned with the transcription of the genetic message into RNA. (1) Clayton and Adler (2) showed that induced catalase synthesis in Rhodopseudomonas spheroides is inhibited by low doses of X-rays, giving experimental support to the idea. Pollard and Vogler (3) using cells in which the process of induction involved both permease and induction showed that there is some sensitivity to gamma radiation. Novelli (4) found a reduced sensitivity as compared with colony formation, but it is still a considerable sensitivity.

The process of induction of an enzyme has been thoroughly studied in recent years, particularly by Pardee and Prestidge, (5), Boezi and Cowie (6) Nakada and Magasanik (7) and Kepes (8). Their work, which supports the well known suggestion by Monod, Jacob and Gros (9), indicates that the transcription of the genetic message is repressed by something which can be acted on by a small molecule, the inducer, to remove repression and permit the formation of messenger RNA, which then acts to make the enzyme. The messenger RNA undergoes decay, by a process which is still not clear. Very elegant measurements by Kepes (8) show that for the messenger RNA for betagalactosidase the half-life is 1.02 min. at 37°C and 2.05 min. at 25°C. The time of onset of the enzyme after induction was found to be about 3 minutes.

If the process of transcription is indeed sensitive to ionizing radiation, then the irradiation of cells which have just been induced should show development of the enzyme to the extent of formation of new messenger RNA within a few minutes plus the formation of the enzyme while the messenger RNA is decaying. This pattern was found by Clayton and Adler. The experiments described here amplify and extend their work, and also permit relatively accurate agreement with the work of Kepes. The experimental procedure is as follows. Cells of E. coli B. or 15 $Thy^{-}Leu^{-}$ are grown in minimal medium on maltose as a carbon source. This does not repress the formation of enzyme, nor does it induce it. When the cells are at a concentration between 5×10^{7} per ml. and 1.0×10^{8} per ml they are induced by the addition of 1 ml of thiomethylgalactoside (TMG) (0.2g/100 ml) to 20 ml cells. The concentration at irradiation must be kept relatively low. At higher concentrations we have found the cells to be much less sensitive for reasons not yet clear.

A few minutes after induction (the time depends on the temperature) the cells are irradiated in a Co^{60} source for an amount of dose ranging about 13,500r, which takes 2½ minutes. In the meantime 1 ml samples are taken at two minute intervals, both from the irradiated and a non-irradiated culture, and assayed for betagalactosidase activity. This is done by putting the 1 ml sample into 4 ml ice cold distilled water containing one drop of toluene and 1 drop of detergent (Sarkosyl, 2%). The samples are shaken vigorously at intervals for one hour, and then placed in a waterbath at 34°C. ONPG (orthonitrophenylbetaDgalactopyranoside, obtained from SIGMA) is added to each tube and the yellow color allowed to develop until it is suitable for reading, the time of assay being recorded. The reaction is stopped with 1M sodium carbonate, and the per cent transmission read on a B and L spectronic spectrophotometer at 420 millimicrons. A calibration curve with known relative amounts of enzyme is used to derive arbitrary units of enzyme activity. The results of one experiment are shown in figure 1. The cells were maintained at 42°C, induced at zero time and irradiated in the cobalt source for the time indicated as shaded. The amount of enzyme produced is plotted for the irradiated culture and also for an unirradiated control. It can be seen that the formation of messenger RNA continues for about 2 minutes after the mid-time of the irradiation, and then ceases. The decay of the messenger, as seen by the slowing down to a stop of the production of enzyme, can be seen. Somewhat later, a reduced rate of synthesis is seen. This, it is suggested, is due to newly synthesized DNA. A separate study of the later increase is going forward.

In figure 2 the same kind of data taken at 20°C is shown. The whole process is considerably slowed down.

If we suppose that the amount of enzyme formed, E, is proportional to the amount of active messenger RNA present, R, and if E_o is the maximum amount observed in the plateau before the second rise, then if we have the decay relation for the messenger RNA,

$$\frac{dR}{dt} = -k_1 R$$

it follows

that $$\ln(E_o - E) - \ln E_o = -k_1 t$$

Thus a plot of the amount of enzyme short of the plateau figure, $(E_o - E)$, versus time, on a logarithmic plot, should yield a straight line, and from it the half-life of the messenger RNA should be deducible. This is the method used by Kepes. In figure 3 are shown such plots for temperatures ranging from 17°C to 45°C. The half-lives and decay constants derived from these graphs are given in table 1, together with data appropriate to other messenger decay studies. It can be seen that the agreement with Kepes is quite good, and the consistency of all the data adds weight to the general idea that radiation has inhibited transcription in something like the same way as the removal of inducer, as studied by Kepes. The mechanism suggested is totally different; only the result is the same.

From figures such as given above one can also derive two other sets of numbers. The first is the time taken to halt transcription. This is measured from the mid-time of irradiation to the time at which the irradiated culture deviates from the control. Values are given in table 2. It is also possible to conduct the same assay on cells which are fully repressed by being grown in glucose. Much less enzyme is observed, but by increasing the time of assay, it can be measured. It has been suggested by

Jacob and Monod (11) that this basal enzyme can correspond to the transcription of one messenger RNA molecule. If we designate the enzyme units measured in a standard way per cell for basal level as one basal unit, then we can find the number of basal units, and hence presumably the number of messenger molecules produced per cell in each case. Two numbers are found : the total number, which will depend on the time of induction and so is not significant beyond the circumstances of the experiments, and the number in the last decay process. This last is significant, as it should be independent of the time of induction. Since it is possible to measure the rate of production of enzyme near the moment of cessation of transcription and also the number of basal units formed after this, we can estimate the rate of transcription in the same units.

This rate of transcription is measured as follows. If we denote messenger RNA by R we have the following relations

$$E = bR$$

$$\frac{dR}{dt} = a - k_1 R \quad \text{until transcription ceases}$$

$$\frac{dR}{dt} = -k_1 R \quad \text{after transcription ceases.}$$

Here E is the amount of enzyme which we measure in basal units, and for convenience we set b = 1, since we do not in these experiments, measure the actual number of enzyme molecules; a is the rate of transcription of messenger per second.

Then we have, at the moment when transcription ceases, an observable value of $\frac{dR}{dt}$ which we call $\left(\frac{dR}{dt}\right)_o$. Also, from the rest of the curve

for synthesis of enzyme we can measure E_o and hence R_o, the amount of messenger left to decay. Thus we have

$$\left(\frac{dR}{dt}\right)_o = a - k_1 R_o$$

and we have measured k_1 as described above. So a can be found.

In table 3 we give numbers found in this way for different temperatures. It is interesting that although the process of stopping transcription is faster at higher temperatures, the rate of transcription is faster still. In figure 4 we show the Arrhenius plots for the decay of messenger and also for the rate of transcription. In the first case the energy of activation, H*, is found to be 15,000 calories per mole, while in the second case it is 18,000 calories per mole. Both are in a reasonable range for enzyme action.

The fact that very reasonable figures for the decay of messenger over a wide range of temperature, with good agreement with good measurements made quite differently, are obtained by assuming that radiation acts to stop transcription, forces consideration of this as a hypothesis. It is not at all unreasonable in view of recent experiments of Pollard and Achey (12) in which it was found that in the presence of oxygen the DNA of E. coli is degraded to the extent of 50% but no more. The process of degradation involves some kind of enzymatic action, and it is here suggested that the 50% degradation occurs on the strand of DNA which is transcribed. This strand can be designated as "physiological". The other strand is supposed to be so held in the cell as to be relatively invulnerable to the combined effect of radiation and enzyme. The kinetics of degradation have been measured at different temperatures in our laboratory, and it is possible to estimate that before the physiological strand

has become 5% degraded, transcription of the message has ceased.

It has been suggested, for quite different reasons, by Champe and Benzer (13), Bautz (14), and McCarthy and Bolton, (15), that only one of the two strands of DNA is transcribed. It is presumably in a different physical state while the process of transcription is going on. It would appear that the difference is sufficient to render the strand which is transcribed much more vulnerable to the combination of radiation and enzymatic attack which is involved in this type of radiation.

Since this type of inhibition of transcription, regardless of the hypothesis advanced to explain it, is not specific at all, it should be possible to exploit it to make a general study of a wide variety of messenger RNA half-lives and also, possibly, of rates of transcription. If one looks at data on the incorporation of sulfur (3), the same kind of cessation of uptake after irradiation is evident. This corresponds to the formation of a quite different, and perhaps more representative protein. Studies specifically directed at the same kind of observations as given above should make it possible to measure half lives of such proteins. This work is being prosecuted.

REFERENCES and NOTES

1. E. Pollard, Amer. Naturalist, 44, 71-84 (1960).

2. R. K. Clayton and H. I. Adler, Biochim. Biophys. Acta, 56, 257-267 (1962).

3. E. Pollard and Vogler, C. Rad. Res., 15, 109-119 (1961).

4. G. D. Novelli, T. Kameyama and J. M. Eisenstadt, Cold Spring Harbor Symposia, 26, 133-145 (1961).

5. A. B. Pardee and L. S. Prestidge, Biochim. Biophys. Acta, 49, 77-86 (1961).

6. J. A. Boezi and D. B. Cowie, Biophys. J., 1, 639-645 (1961).

7. D. Nakada and B. Magasanik, J. Mol. Biol., 8, 105-127 (1964).

8. C. Levinthal, A. Keynan and A. Higa, P.N.A.S., 48, 1631-1638 (1962).

9. A. Kepes, Biochim. Biophys. Acta, 76, 293-309 (1963).

10. J. Monod, F. Jacob and F. Gros, Biochem. Soc. Symp., 21, 21-38 (1961).

11. F. Jacob and J. Monod, J. Mol. Biol., 3, 318-24 (1961).

12. E. Pollard and P. M. Achey, Science, 140 000 (1964).

13. S. P. Champe and S. Benzer, P.N.A.S., 48, 532-545 (1962).

14. E. K. F. Bautz, P.N.A.S., 49, 68-73 (1963).

15. B. J. McCarthy and E. T. Bolton, J. Mol. Biol., 8, 184-200 (1964).

16. It is a pleasure to thank Dr. Richard Wax for many useful suggestions.

17. Many of the assays were most competently done by Mrs. Kathy Kolacz. Her help is gratefully acknowledged.

18. The work was supported by N.A.S.A. Contract NsG 324.

CAPTIONS FOR FIGURES

Figure 1. A culture of E. coli, strain B previously grown on maltose was induced with TMG at time zero. It was then allowed to grow at 42°C and irradiated in a Co^{60} source for 2¼ minutes, giving a dose of 13,500r. The amount of betagalactosidase in 1 ml of the culture was measured at various times for this culture and an unirradiated control. The production of enzyme continues for a short while after irradiation and then ceases. From the kinetics of cessation of production the half-life of messenger RNA can be found. Later, presumably as new DNA synthesis starts, the supply of enzyme begins to increase.

Figure 2. Data similar to Figure 1 with the temperature at 20°C.

Figure 3. Plots of the amount of enzyme short of the plateau value versus time on a semilogarithmic scale. The decay character is clearly visible, and the half life of messenger RNA can be found for various temperatures.

Figure 4. Plots of the logarithm of the decay constant for messenger RNA and the rate constant for transcription of message versus the reciprocal of the absolute temperature. The activation energy for decay is 15,000 cal. per mole, while that for transcription is 32,000 cal. per mole.

TABLE 1

Constants for the Decay of Messenger RNA at Different Temperatures.

Temperature, degrees C.	1/Kelvin Temperature	Half-life	Decay constant k_1	log k_1
17	3.45×10^{-3}	5.2 min.	1.3 min^{-1}	0.115
20	3.42	4.2	1.6	0.205
25	3.35	1.3 2.4 1.9 2.05 (Kepes)	3.6	0.560
30	3.31	2.5 (Nakada and Magasanik)		
37	3.23	0.80 0.75 1.05 (Kepes)	9.0	0.952
42	3.18	0.8	8.6	0.935
45	3.14	0.56	12.4	1.095

Levinthal, Keynan and Higa, B. Subtilis, 33.5°C, found a half-life of 1.4 min.

TABLE 2

Time of Cessation of Synthesis of Messenger RNA after the Mid-time of Irradiation

Temperature, ^{o}C	Time, min.
17	7
20	7.5
25	4.5 3.5 2.5
37	1.5
42	1.5
45	2.7

TABLE 3

Amounts of Enzyme Synthesized after Cessation of Transcription, and Rates of Transcription. Units are "quotas" Produced by Repressed Cells, and Possibly Messenger RNA Molecules per Cell.

Temperature, ^{o}C	Quotas made after cessation of transcription.	Rate of transcription, messenger quotas per minute.
17	1.5	0.38
20	0.5	0.14
25	1.6	1.6
37	3.7	6.2
42	3.0	6.3
45	8.9	21.2

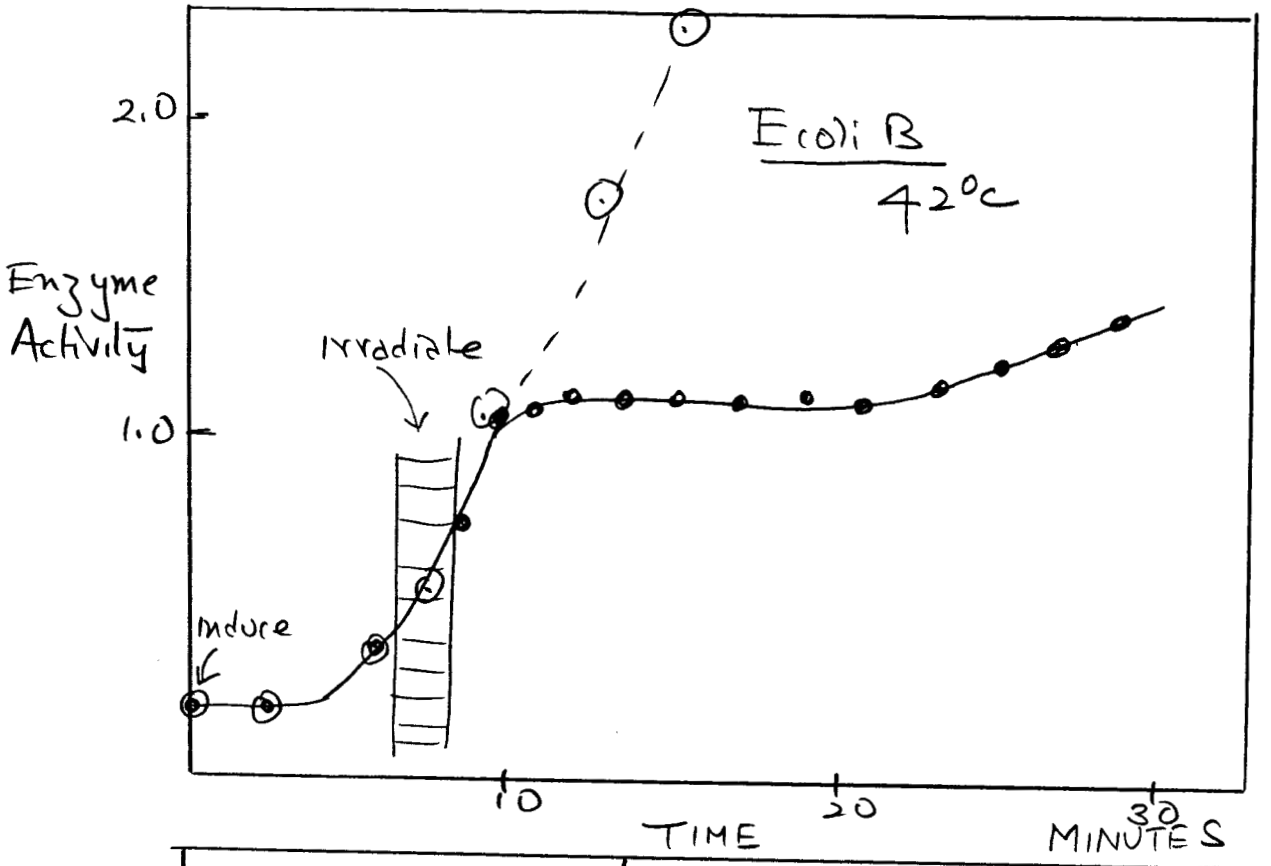
2.0
1.0
Enzyme Activity
E coli B
42°C
Irradiate
Induce
10
20
30
TIME
MINUTES

Fig 1

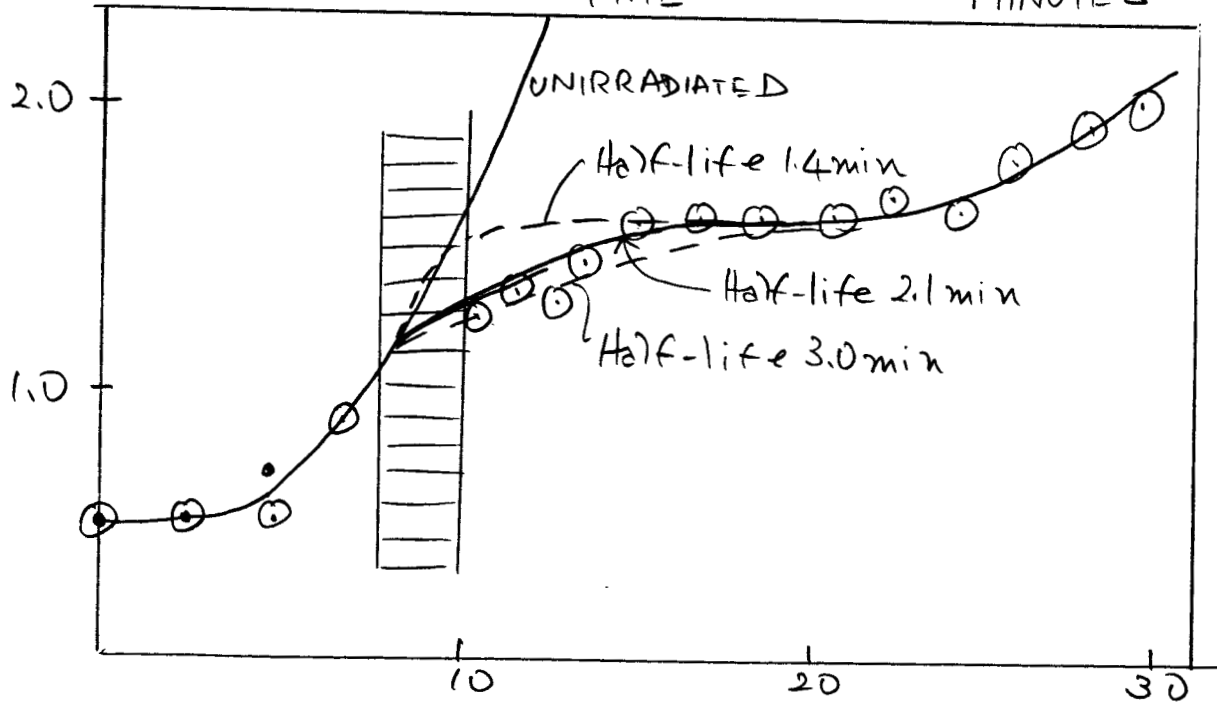
2.0
1.0
UNIRRADIATED
Half-life 1.4min
Half-life 2.1 min
Half-life 3.0min
10
20
30

Fig 2

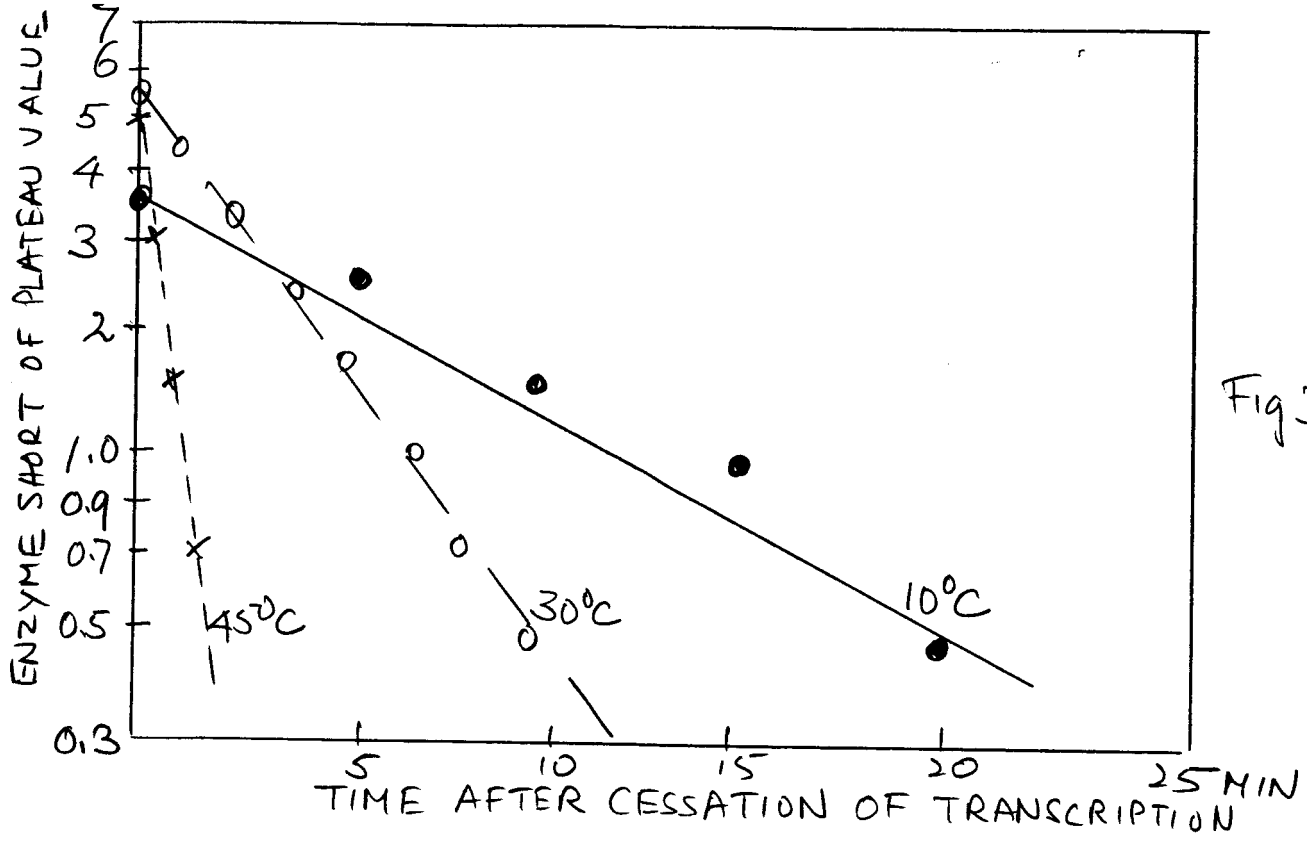
ENZYME SHORT OF PLATEAU VALUE
7
6
5
4
3
2
1.0
0.9
0.7
0.5
0.3
45°C
30°C
10°C
5
10
15
20
25 MIN
TIME AFTER CESSATION OF TRANSCRIPTION

Fig 3

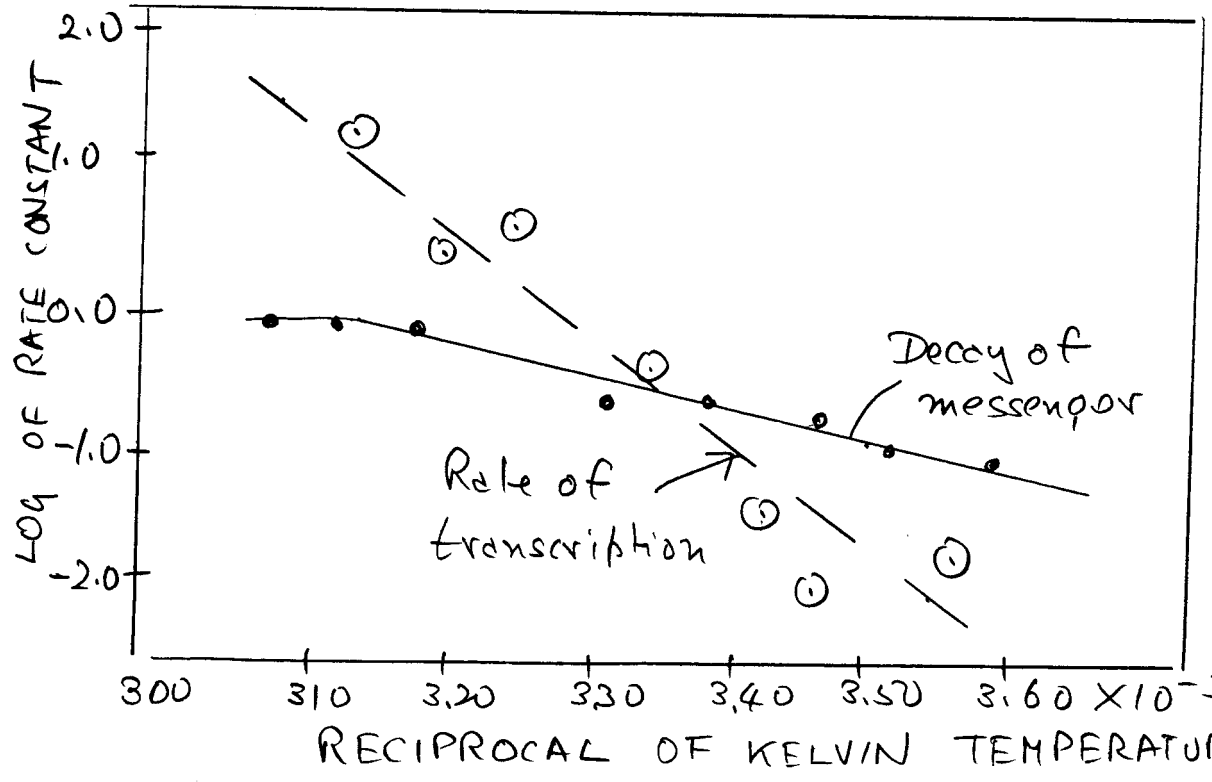
LOG OF RATE CONSTANT
2.0
1.0
0.0
-1.0
-2.0
Decay of messenger
Rate of transcription
300
310
3.20
3.30
3.40
3.50
3.60 X10⁻³
RECIPROCAL OF KELVIN TEMPERATURE

Fig 4

N65 10114

Rate of Mutation to Phage Resistance in D_2O Medium

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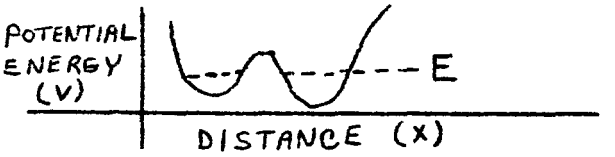
It has been suggested, notably by Freese (1) that a change in a base pair in nucleic acid from an adenine-thymine pair, to a guanine-cytosine pair or vice-versa, constitutes a process of mutation. A further suggestion has been made by Per-Olov Owdin (2) that this change could be mediated by the tunneling of a proton from one base to another. The process of proton tunneling involves the passage through a potential barrier, and the probability of passage P is given by an expression of the form below

$$P = Ae^{-\int \frac{2\pi}{h}\left\{2m(E-V)\right\}^{\frac{1}{2}} dx}$$

where A is a constant, m is the mass of the tunneling particle, h is Planck's constant and E, V and x are as indicated in figure 1.



The tunneling process involves the wave length of the particle which does the tunneling, and this in turn involves the mass of the particle. If the particle is a proton then it has one-half the mass of a deuteron and for a given situation the probability of tunneling will change by introducing in the exponent a factor of the square root of 2, which will materially change the absolute magnitude of the quantity

involved. If we suppose that the probability for mutation due to this effect is about one in a million, and if this is due to the tunneling of a proton, then the presence of a deuteron would cause a change by a factor from 10^{-6} to approximately 10^{-9}. Thus if it should be true that the sole cause of mutation was proton tunneling in DNA, and it were possible to make a full exchange of proton for deuterons, the mutation rate should drastically fall for bacteria which had been cultured in D_2O medium.

It is readily possible to grow E. coli cells in D_2O medium and it is possible to measure the process of mutation to phage resistance, both for T1 and T2r phage. All that is necessary is to plate the bacteria on medium after they have been challenged by phage, and the mutants appear as resistant colonies.

The question arises as to whether cells grown on medium in which the components are of normal hydrogen while the water is heavy water, do have appreciable substitution of deuterium for protium. In our own laboratory we can offer the following evidence. T1 phage produced by infection of cells grown on D_2O medium (normal nutrient broth made in D_2O) shows markedly different thermal inactivation from phage grown on H_2O grown cells. This indicates a real change has been made in a phage which has to be formed from the metabolic operations of the cell. Also betagalactosidase from cells grown in D_2O medium has different thermal inactivation constants. We conclude, as is reasonable, that the biochemical processes which involve water cause the incorporation of deuterium in place of normal hydrogen.

A certain amount of care must be exercised in this kind of study, because mutation to phage resistance may not immediately alter

the surface of a bacterium in such a way as to prevent an attachment of phage, which is the basis for the blocking of the infective process. Thus one must allow sufficient time for the mutation to become expressed as phage resistance.

Procedures

Cells of Escherichia coli, strain B (ATCC 11301) were grown in nutrient broth, with agitation overnight. In the morning 100 ml of fresh nutrient broth were inoculated with 1 ml of the overnight culture and allowed to grow for about an hour before sampling. Every half hour 7 ml were taken, and used to measure (a) turbidity, (b) dilution and plating for cell colony count and (c) challenge for mutants.

This last was done by adding 2 ml of phage at 10^9 per ml to 2 ml of cells, allowing to stay mixed for two hours, diluting and plating by the agar overlay method with an additional ½ ml of phage added to the tube agar before pouring on the plate.

The plates were incubated overnight at 37°C and mutant colonies counted.

The same procedure was used for D_2O cells which were grown in nutrient broth dissolved in D_2O, first overnight and then transferred.

A comparable set of experiments were done in Roberts' C minimal medium (NH_4Cl, 2g; Na_2HPO_4, 6g; KH_2PO_4, 3g; NaCl 3g; $MgCl_2$, 0.01g; Na_2SO_4, 0.026, glucose 6g per liter).

Typical data are shown in Figure 1 and Figure 2. In these experiments the colony count of the growing cell culture is shown as one line, the number of mutants to T1 resistance is shown as a second line and the number of mutants to T2 resistance is shown as a third line.

Similar data taken in D_2O medium is shown in Figure 2. It can be seen at once that there is no striking difference in the number of mutants.

In Figure 3 we show the number of mutants per bacterial cell as the culture develops. The overall progress of the development of mutants can be seen in each case here shown for T1. In figure 4 similar data for T2 are shown. Table 1 shows the number of mutants per cell at each time for each kind of medium.

Experiments done in C minimal medium showed a higher overall rate of mutation, but no systematic difference between D_2O and H_2O. Only T1 resistance was studied.

Cultures which had been stored overnight in D_2O and H_2O did not show any appreciable difference in the number of mutants.

DISCUSSION.

We conclude from this preliminary experiment that it is not safe to assume that the only basis for the formation of mutants is the process of proton tunneling. We do not claim that in these experiments we have eliminated it as a means of causing mutations, but merely suggest that the discovery of mutants due to proton tunneling might be done by this means. We also suggest that it will be found to be rare compared with other mechanisms which are responsible for the formation of mutants. Our data could be interpreted as showing a smaller mutation rate in D_2O medium. It is likely that this reduced rate is due to other factors which are known to influence mutation expression, but if the claim were made that this reduction is due to reduced proton tunneling, then the ratio of 0.6 ± 0.2 for T2 and 0.7 ± 0.3 for T1 between D_2O medium mutants and H_2O medium mutants should be compared with theoretical tunneling ratios of 1.4×10^{-3} and 6.5×10^{-3}. If we suppose that a fraction f

of mutants is due to tunneling, and 1-f is not dependent on D_2O in any other way, then the observed ratios can be used to calculate f, using the theoretically expected ratio as given above. We get for the calculated ratio

$$\frac{D_2O}{H_2O} = \frac{1.4 \times 10^{-3} f + 1-f}{1} \quad \text{for T2 and}$$

$$\frac{6.5 \times 10^{-3} f + 1-f}{1} \quad \text{for T1}$$

Thus f is 40% in the first case and 30% in the second. Since it is very likely that the assumption that 1-f does not depend on D_2O is not valid, the value of f calculated above is probably too high. For this reason we feel it is safe to conclude that other mechanisms of mutation exist.

ACKNOWLEDGEMENTS

We wish to thank Dr. Stanley Person for advice and criticism.

REFERENCES

1. Freese, E. Proc. Nat. Acad. Sci. Wash. 45 622 (1959).

2. Owdin, Per-Olov Rev. Mod. Phys.

TABLE 1

Mutants per cell for T1, T2 resistance in H_2O and D_2O media.

Time	T1(H_2O)	T1(D_2O)	T2(H_2O)	T2(D_2O)
0	4.6×10^{-6}	2.9×10^{-6}	0.7×10^{-8}	Not observed
1h	4.5×10^{-6}	3.2×10^{-6}	2.2×10^{-8}	0.4×10^{-8}
2h	3.3×10^{-6}	3.4×10^{-6}	7.0×10^{-8}	3.6×10^{-8}
3h	2.4×10^{-6}	2.9×10^{-6}	7×10^{-8}	4.1×10^{-8}
4h	2.1×10^{-6}	2.2×10^{-6}	8.5×10^{-8}	7.0×10^{-8}
5.5h	--	2.5×10^{-6}	--	5.1×10^{-8}

NGS 10115

THE NATURE OF BACTERIAL REVERTANTS PRODUCED BY THE DECAY OF INCORPORATED TRITIATED COMPOUNDS.

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Introduction

It has been shown that tritiated thymidine decay in bacteria produces mutations as well as lethal events (Person and Lewis,1962). Using tritium labeled precursors for RNA,DNA and protein, the effeciency for reversion production is in the following order: 7(for H_3-uracil) to 2(for H_3-thymidine) to 1(for H_3-histidine or H_3-proline (Person and Bockrath,1964). In view of the work by Rachmeler and Pardee (1963) and Person (1963) that shows the order for effeciency of producing lethality to be: 2(for H_3-uracil) to 3(for H_3-thymidine) to 1(for H_3 histidine or H_3-leucine), it is unexpected to note the relatively high reversion frequency produced by the decay of incorporated H_3-uracil.

With the idea that the revertants produced by the decay of different H_3 compounds may be different, we have examined in some detail revertant production as a function of the growth conditions following the accumulation of H_3 decays.

Materials and Methods

A multiple auxotroph of E. coli. strain 15[1] requiring thymidine, uracil, tryptophan, methionine, agranine and proline was grown in supplemented minimal salts - glucose medium to a titer of about 5×10^8 and diluted 100x into prepared culture tubes. Each contained the four required

amino acids at a concentration of 50mg/ml, thymidine at 2-3 mg/ml and uracil at 15-20 mg/ml (or uradine at 30-40 mg/ml).

The tritium isotope was incorporated via H_3-thymidine[2] at a final specific activity of 1-2C/mM, H_3-uracil[2] at a final specific activity of about 0.2 C/mM, or H_3-histidine[3] at a specific activity of approximately 1C/mM and concentration of 2-3mg/ml. The inoculated cultures, aerated at 37°C, grew exponentially at indistinguishable rates with a doubling time of approximately 45minutes. When the cell count reached 5-7 x 10^8 cells per ml, the cultures were generally harvested by filtration, washed and re-suspended in minimal salts - glucose- and stored at 4-8°C to accumulate radioactive decays.

The rate of decay per bacterium of incorporated tritium compounds was ascertained by radioactivity determinations using liquid scintillation techniques[4] and cell number measurements[5] of equal aliquots from the re-suspended stored cultures. The observed counts per minute in the radioactive aliquots were corrected for counter efficiency and self absorption of the beta-particle by the bacterium, and then divided by the number of bacteria per aliquot to give the real decays per unit time per bacterium.

Viable titer and revertants were determined by colony counts on minimal salts - glucose agar pettri dishes supplement in the following manners: a)the titer plates contained 1 mg/ml Difco nutrient broth; b)the fortified revertant search-plates contained 100 mg/ml each of tryptophan, methionine and proline and 20 mg/ml each of uracil and thymidine ------- plus 200 mg/ml Difco nutrient broth and the non-fortified search-plates (independent revertant) contained the five supplements at concentrations just listed, but no nutrient broth fortification.

Results

The dependent and independent subclasses in revertant population.

If the labeled cultures are allowed to accumulate decays the number of viable arginine revertants, forming colonies on fortified search plates, increases to a maximum in the region of 100-150 decays per bacterium. This is shown for atypical H_3-uracil labeled culture in figure 1. The examination of the revertant population utilized this maximum number of viable revertants (indicated by the shaded area) by allowing 100-150 decays per bacterium to accumulate in the H_3-thymidine, H_3-uracil, H_3-histidine labeled cultures.

After sufficient time for such a number of decays to accumulate, the cultures were removed from cold-storage and incubated at 37°C with aeration in the presence of tryptophan, methionine, proline, thymidine, uridine, and a low level of nutrient broth fortification. the supplements and nutrients were available in the same concentrations as those in the fortified search plate. At intervals in time aliquots were removed from the incubation tubes and assayed for revertants on fortified and non-fortified (independent revertant) search plates. The results are shown in figure 2.

The number of revertant colonies indicated on the fortified search plate remains constant for about two hours in the revertant populations produced by H_3-thymidine or H_3-histidine decay, or three to four hours in the case of H_3-uracil.

At the same time the number of independent revertant colonies formed on non-fortified search plates is initially quite low. After a slight lag this number increases exponentially to eventually equal the number obtained on the fortified plate. It appears that the incubation increases the number of independent revertants which can, in the absence of continued nutrient fortification, give rise to a revertant colony. This is accompanied by no substantial variation in the over all titer and a slight increase in that number of revertant colonies developed on a fortified plate.

The differential response of the revertant population when plated on fortified and non-fortified Petri dishes is taken as evidence for the existence of two subclasses of revertants produced by H^3 decay: the independent revertant forms a colony in the absence of fortification while the dependent revertants form additional colonies in the presence of fortification. These two subclasses are shown to exist for all three H^3 compounds used, although the kinetics of development differ.

Figure 3 shows the specific variations of the independent fractions in the different revertant populations as a function of incubation time. The ratio of the number of revertant colonies on non-fortified plates over the number of revertant colonies on fortified plates (from fig.2) is plotted against time of incubation. At zero incubation time the independent fraction in the cultures having accumulated decays from incorporated H^3-thymidine or incorporated H^3-histidine is about 5 times that found in the culture receiving decays from incorporated H^3-uracil. (See table 1*(a)*) At three hours the revertant populations in the H^3-thymidine and H^3-histidine cultures are almost entirerly composed of independent revertants, whereas more than four hours are required for the H^3-uracil culture to reach such a distribution. This seems to be the result of similar rates of conversion in the three cultures, but dissimilar initial distributions.

<u>"Independent revertants as a function of accumulated H^3 decays"</u>.

A second set of experiments was undertaken to demonstrate that the independent fraction of the revertant population was composed of a particular type of revertant, having a distinct probability of being produced per tritium decay.

At intervals in the accumulation of decays large aliquots from each of three labeled cultures (H^3-thymidine, H^3-uracil, H^3-histidine) were removed from cold storage and plated directly on to non-fortified search plates. At

the same time viability was determined on titer plates. The results of several experiments are shown in figure 4. The number of revertants produced by decays is plotted as the number of viable revertants per 10^8 viable bacteria vs the number of accumulated decays.

The data shown in figure 4 represent the net number of revertants produced by the accumulation of tritium decays during cold storage. Two types of "background" revertants have been discounted. The first is merely the number of spontaneous revertants that appear among a similar number of unlabeled bacteria. The second type includes those revertants that are produced by tritium decays occuring during the labeling process. That is, per bacterium plated, at the beginning of cold storage, the number of revertants is greater for labeled cultures than for unlabeled control cultures. Both types of "background" revertants are adjusted to account for the increasing loss of colony forming ability accompanying accumulating tritium decays.

The lines drawn in figure 4 are fitted to the data by the least square method. The slopes of these lines represent the reversion frequencies (termed k values) in units of revertants per 10^8 survivors per tritium decay. These k values are 0.058, 0.026 and 0.019 for H^3 thymidine, H^3-uracil and H^3-histidine respectively.

Comparison of data from figures 2,3 and 4.

Table 1 is used to compare data taken from figures 2,3 and 4. The first line in table 1 (a) lists the fraction of independent revertants in the total revertant populations as determined by zero time extrapolations of data in figure 3. In the second line these fractions are normalized to H^3-uracil taken as 1.0

The first line in table 1 (a) lists the k values determined by

the H^3-thymidine, H^3-uracil and H^3-histidine experiments of figure 4. Line 2 lists k-values obtained previously by plating on nutrient broth fortified Petri dishes (1964). Since line 1 represents k values for independent revertants, the ratio of independent/total revertants may be formed and this result is indicated in line 3. In the last line of table 1 (a) these ratios are also normalized to H^3 - uracil taken as 1.0

~~Table 1 (b):~~ The normalized ratios in parts (a) and (b) of table 1 show clearly that decays of incorporated H^3-uracil yeild a relatively small fraction of independent revertants. This is found in the detailed study of revertant populations resulting from a large accumulation of tritium decays and in the reversion frequencies accompanying decays.

It should be noted, however, that the absolute magnitude of the independent revertant fractions are not the same when determined by the two methods. That the ratios determined by ~~comparison~~ reversion frequency k-values are uniformly lower than those found by sub-class development studies could be caused by the different procedures used. While the data in figure 4 used aliquots of cold, stored cultures plated in the cold without the fortified warming, the platings for data shown in figures 2 and 3 took place after cold, stored cultures were fortified with nutrient broth and supplements and warmed to 37°C.

Discussion:

There seem to be two types of revertants produced by H^3- decay. The majority are dependent revertants which give rise to revertant colonies in the presence of nutrient fortification. In addition, there are some independent revertant colonies that can form colonies in the absence of nutrient fortification. The data from figures 2 and 3 show that these two subclasses exist and that they exist for all H^3- compounds used. However, as indicated in figure 2, the kinetics of development of independent revertants varies for the H^3 compounds used, being similar for H^3-thymidine and H^3-histidine and different for H^3-uracil. In addition, the data graphed in figure 3 shows that the fraction of independent revertants in a total population of revertants varies depending upon the H^3- compound. The decay of H^3-thymidine or H^3-histidine yeilds a relatively higher fraction of independent revertants (3 - 5x) than does the decay of H^3-uracil.

If only the production of independent revertants is considered (Fig. 4) the relative mutagenic effeciency of the 3 H^3 compounds used is in the order: thymidine,3, to uracil,1.4, to histidine ,1.0 (Fig. 4). This order of effectiveness is the same as that found for the production of loss of viability (Person,1963; Rachmeler and Pardee, 1963; Person and Bockrath, 1964), with only slight diffrences in absolute relative frequencies. This would suggest that the same molecular species is being perturbed in both cases, and that, in fact, the species is DNA. The independent revertants may result from DNA alterations that require no cell synthesis prior to expression.

If one assumes that the fortified search plate develops colonies equally well from independent and dependent revertants, the number of colonies scored on such a plate would represent the sum of these two subclasses of revertants. The dashed line in figure 2 represents this theoretical sum varying as though it were composed of a constant number

of dependent revertants plus the increasing number of independent revertants (arising by division of pre-existing independent revertants). Since the experimental line obtained is notably below the theoretical line, the increasing number of independent revertants must be accompanied by a decreasing number of dependent revertants. Hence it seems that the population of revertants produced by accumulated H^3-decays is in part composed of dependent revertants which require fortification for expression, and in part composed of independent revertants which do not. And in the course of incubation dependent revertants convert to independent revertants.

We have tried arginine as a substitute for the nutrient broth fortification and find 2 µg/ml to give results similar to those reported above. This would suggest that expression of dependent revertants may require only re-initiation of normal protein synthesis by a temporary exogenous supply of the missing metabolite. In this sense production of revertants by H^3- decay would be different from reversion by U.V. irradiation, where nutrient broth and a particular state of protein synthesis is required for expression (Witkin,1956).

In any event the unique character of the H^3-uracil produced revertant population relative to revertant populations produced by H^3-thymidine or H^3- histidine infers that there are at least two different molecular alterations that can mediate the reversion of a bacterial mutant, and that the specificity of the 3 H^3-compounds used is sufficient to differentially effect these two alterations.

Bibliography

Person, S. Biophysic. J., 1963, 3,183.

Person, S. and Bockrath, R.C.,gr., Biophysic. J., 1964, 4 (Sept.).

Person, S. and Lewis, H.L. Biophysic. J., 1962, 2, 451.

Rachmeler, M. and Pardee, A.B. Biochem et Biophysic. Acta, 1963, 69,62.

Witkin, E. Cold Spring Harbor Symp. Quant. Biol.,1956, 21,123.

Legends for Figures

Figure 1. The curve in figure 1 is the result of production and survival of arginine revertant bacteria. The number of surviving revertants is plotted as a function of accumulated H^3-uracil decays. The examination of the revertant population, as shown in figure 2, utilized the maximum number of viable revertants. This is indicated by the shaded area in the region 100-150 decays/bacterium.

Figure 1

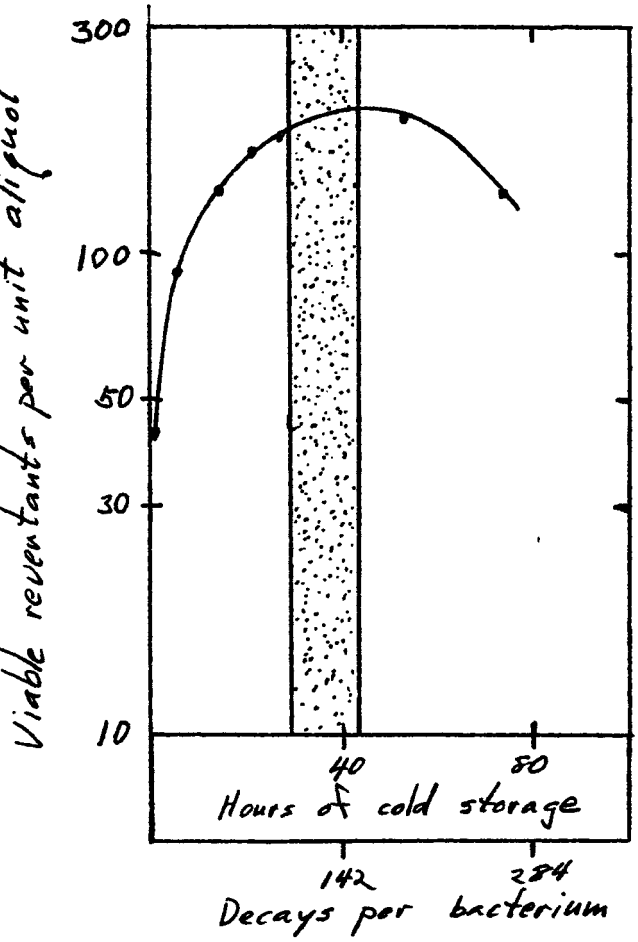

300
100
50
30
10
Viable revertants per unit aliquot
40
80
Hours of cold storage
142
284
Decays per bacterium

Figure 2. The differential response of the different revertant populations, shown here, when plated on fortified and non-fortified Petri dishes is taken as evidence for the existence of two sub-classes of revertants produced by H^3-decay. Aliqouts of stored cultures (see fig. 1) were incubated with nutrient broth fortification and plated as a function of time of incubation on the two types of search plates. The upper solid curves correspond to plating on fortified search plates and the lower solid curves to plating on non-fortified search plates. Independent revertants form colonies in the absence of nutrient fortification while the dependent revertants form additional colonies in the presence of fortification. Every point represents the average of three experiments.

Figure 2

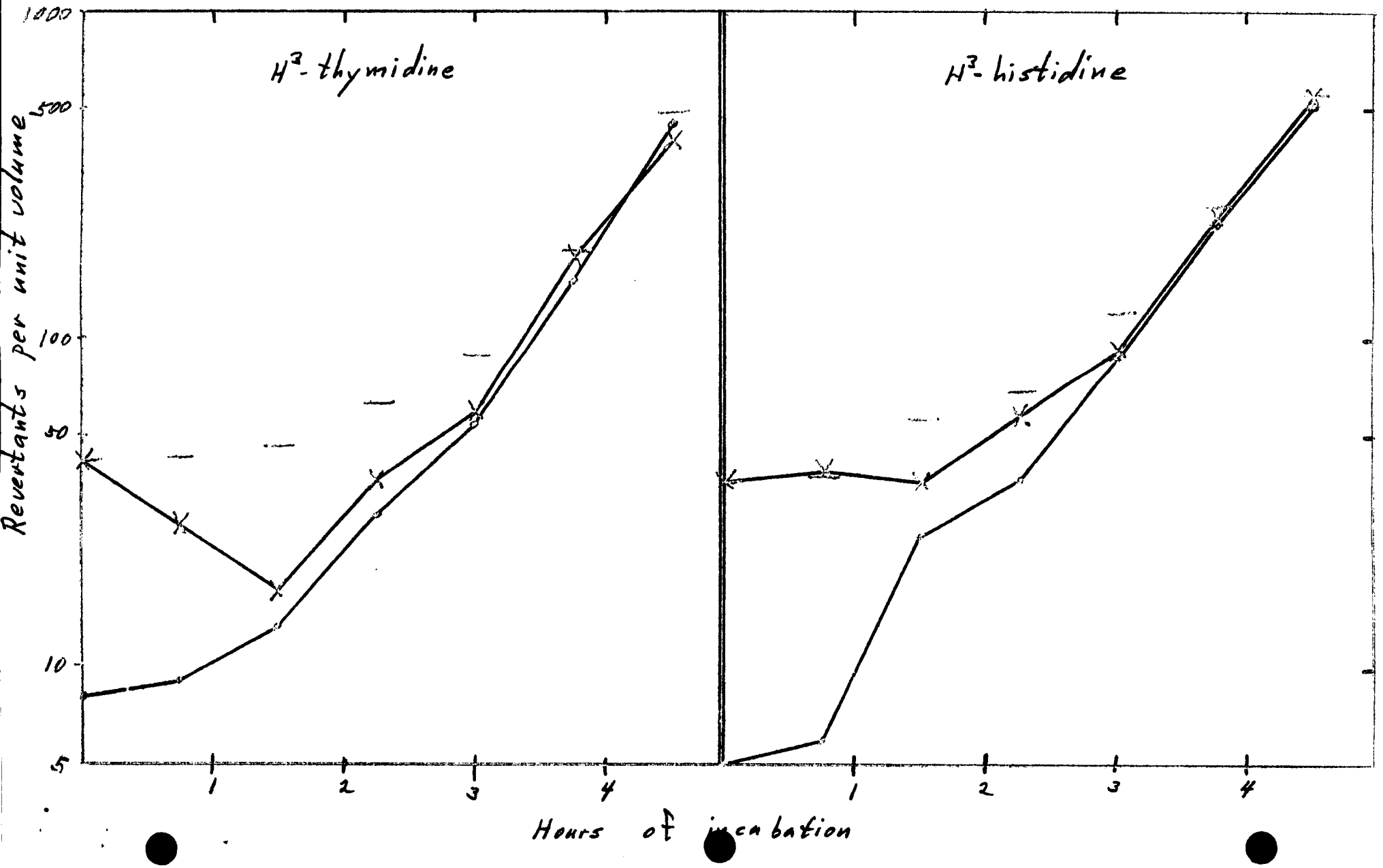

H^3-thymidine
H^3-histidine
Revertants per unit volume
1000
500
100
50
10
5
1
2
3
4
Hours of incubation

Figure 2

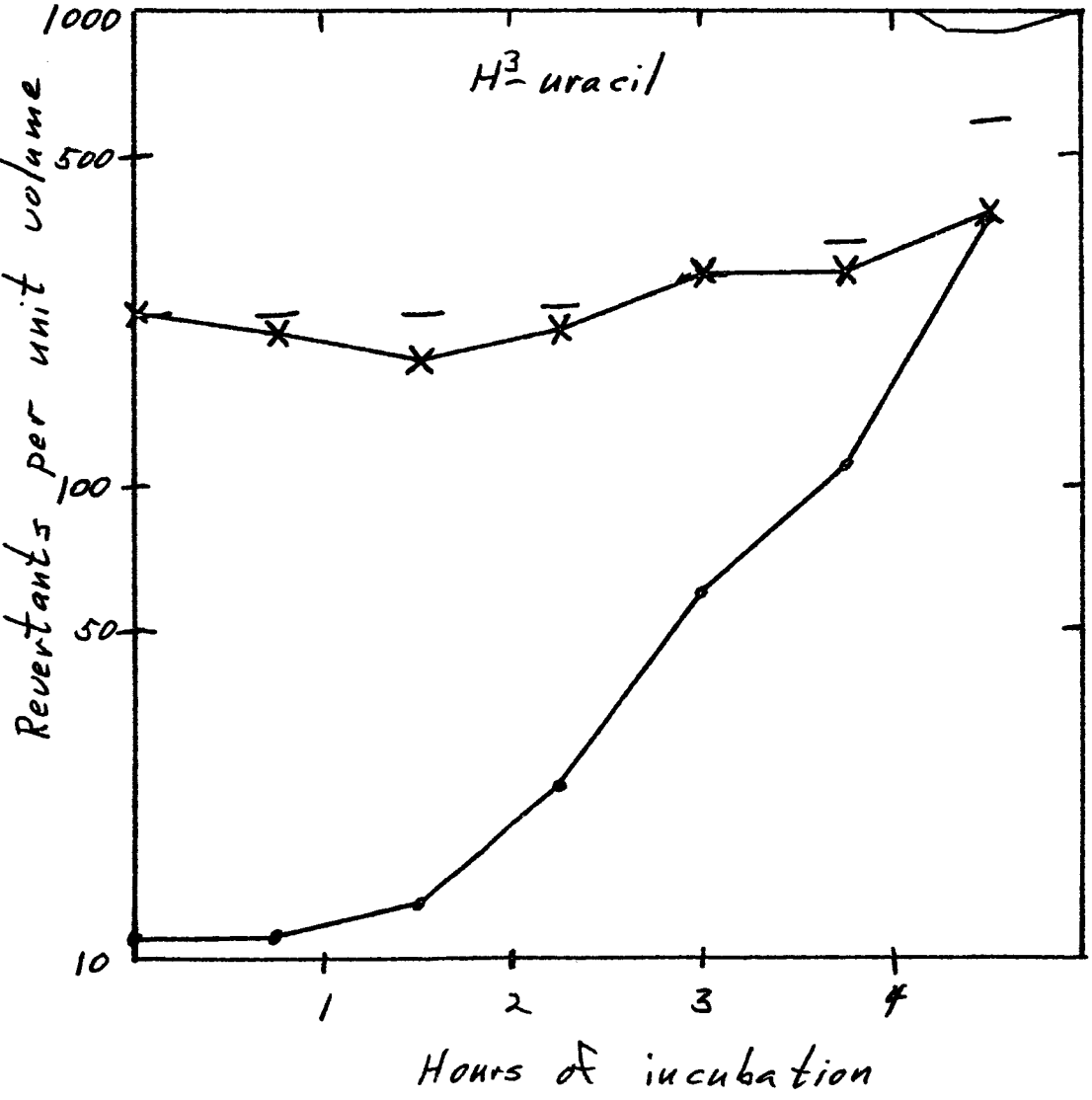

1000
500
100
50
10
H^3 uracil
Revertants per unit volume
1
2
3
4
Hours of incubation

Figure 3. Figure 3 shows the specific variations of the independent fractions in the different revertant populations as a function of incubation time. The ratio of the number of revertant colonies on non-fortified plates over the number of revertant colonies on fortified plates (from fig.2) is plotted for H^3-thymidine, H^3-uracil and H^3-histidine produced revertants. The variation in the fraction of independent revertants over time is similar for H^3-thymidine and H^3-histidine but different for H^3-uracil.

Figure 3

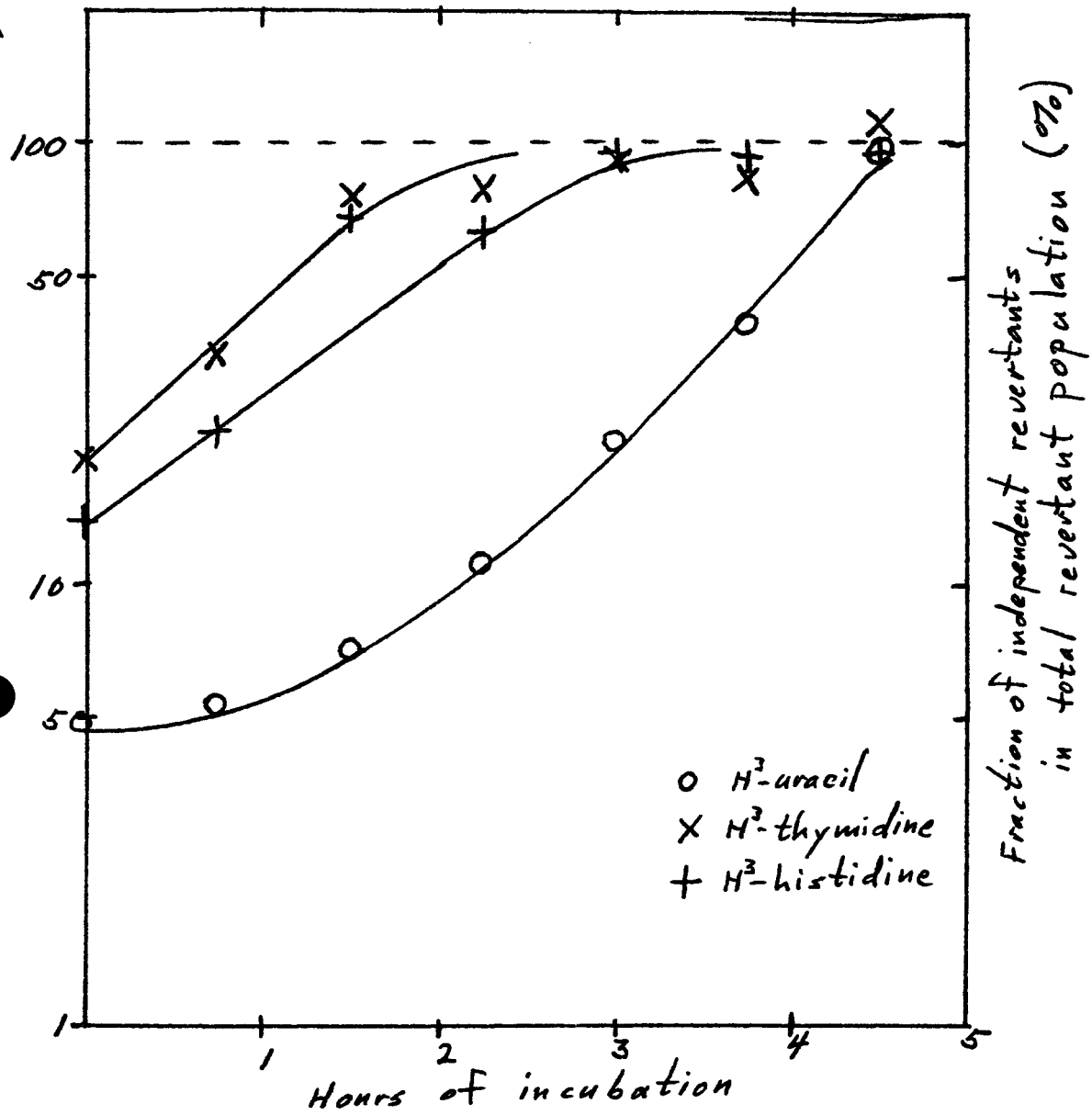
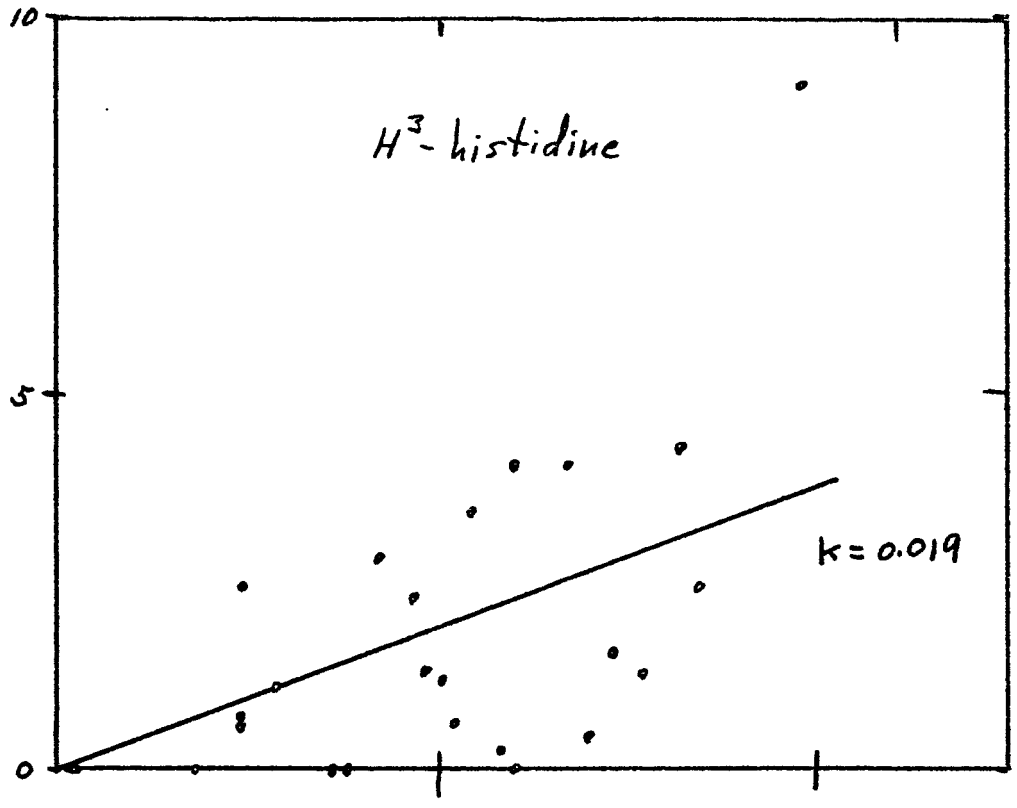
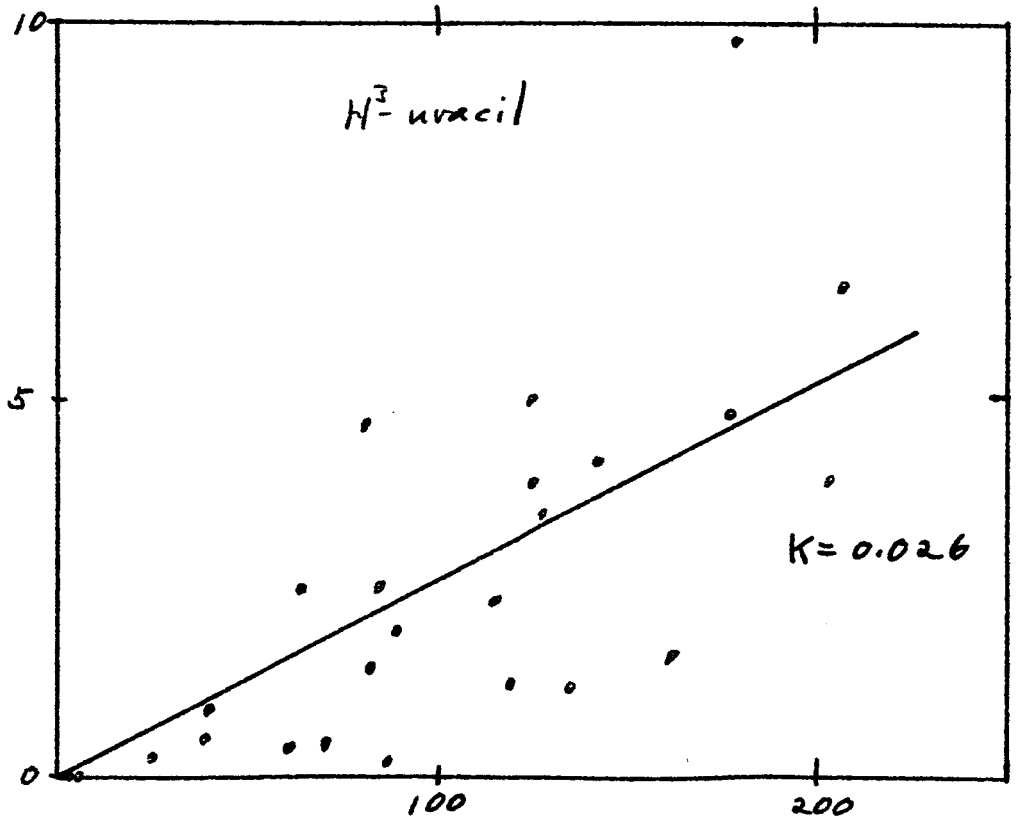

100
50
10
5
1
1
2
3
4
5
Hours of incubation
Fraction of independent revertants in total revertant population (%)
o H³-uracil
× H³-thymidine
+ H³-histidine

Figure 4. Independent revertants as a function of accumulated H^3-decay: The number of viable revertants per 10^8 viable bacteria is plotted as a function of accumulated decays. the lines drawn are fitted to the data by the method of least squares. The slopes of these lines represent the reversion frequencies (k values) in units of revertants per 10^8 survivors per H^3-decay. These k values are 0.058, 0.026 and 0.019 for H^3-thymidine, H^3-uracil and H^3-histidine, respectively.

Figure 4





Reventants per 10^8 bacteria

Figure 4 – H^3 Thymidine

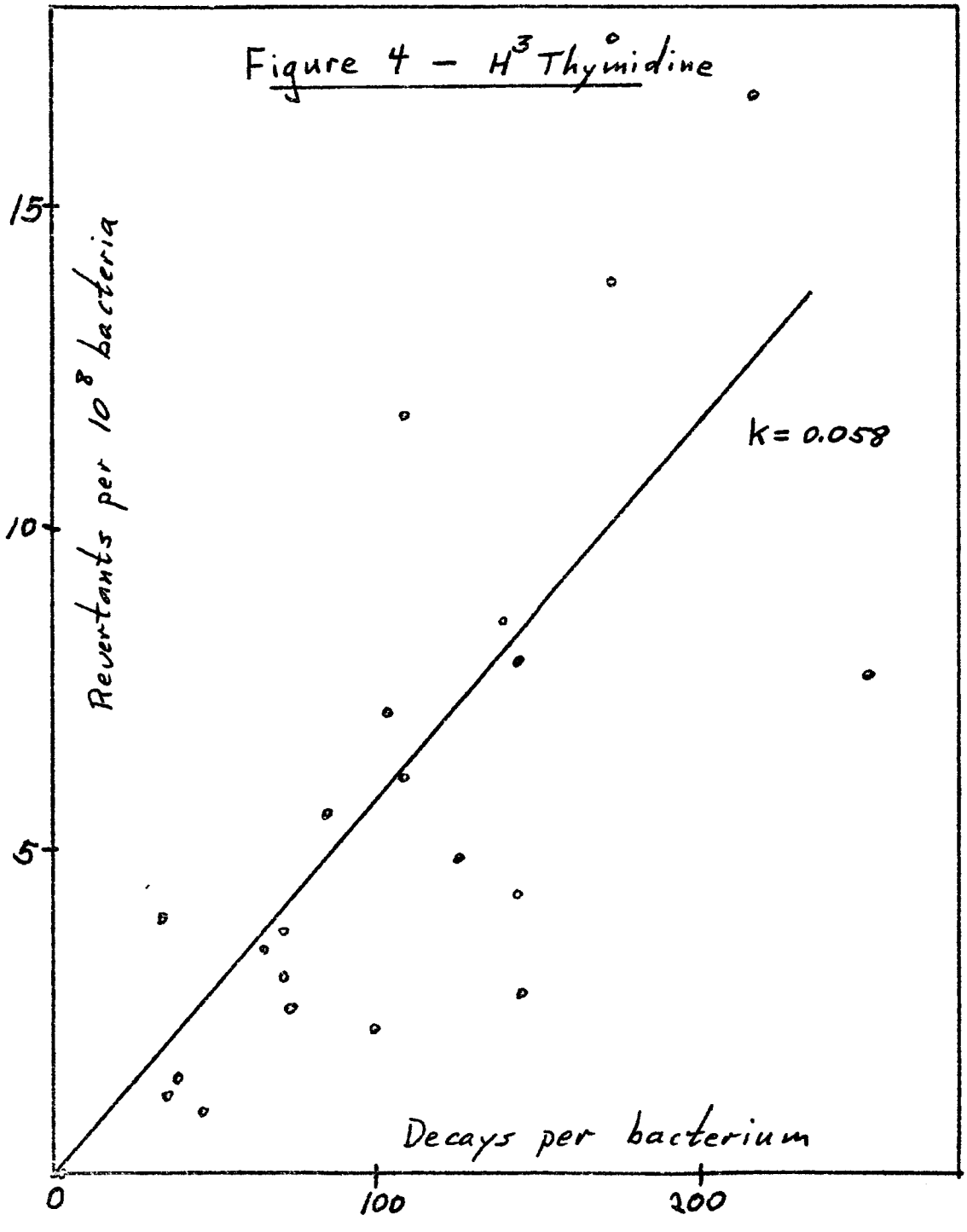


Table 1

Labeling compound	H^3-thymidine	H^3-uracil	H^3-histidine
(a)Ratio of: independent / total	0.19	0.048	0.14
Normalized ratio	4.0	1.0	2.9
(b)Independent revertants*	0.058	0.026	0.019
Total revertants	0.67**	1.84**	0.28**
Ratio of: independent / total	0.087	0.014	0.068
Normalized ratio	6.2	1.0	2.9

* per 10^8 survivors per decay
** determined previously (1964)

Table 1 (a): The ratio of independent revertants to the total revertants as seen at zero time in figure 3 are listed and normalized for convenience of comparison.

Table 1 (b): the reversion frequencies for independent revertants by the decay of incorporated H^3-thymidine, H^3-uracil, or H^3-histidine, as determined in figure 4, are listed, with reversion frequencies for total revertants as determined previously (Person and Bockrath, in press). The ratio of reversion frequencies are calculated and normalized for convenience of comparison.

Summary:

The existence of two sub-classes of revertants has been demonstrated among the revertant populations produced by H^3-thymidine, H^3-uracil and H^3-histidine decays. The majority of the total revertant population is composed of dependent revertants which give rise to revertant colonies in the presence of nutrient fortification. In addition, there are some independent revertants that can form colonies in the absence of nutrient fortification. The relative mutagenic effeciency for production of independent revertants is in the order: H^3-thymidine,3, H3-uracil, 1.4,H^3-histidine, 1.0. We have previously shown that independent plus dependent revertents are produced with efficiencies in the order:H^3-uracil, 6.7, H^3-thymidine, 2.4, H^3-histidine, 1.0. All these data infer that there are at least two molecular alterations that can mediate the reversion of a bacterial mutant by H^3-decay and that the specificity of the H^3=compound used is sufficient to differentially effect these two alterations.

Appendix 4.

Problems of Microspectrophotometry.

The design of a microspectrophotometer depends largely upon the intended use of the instrument. For investigations of proteins and nucleic acids with mainly ultra-violet absorption, a u-v light source and quartz or reflecting optics must be used. Normally this means a rather heavy power supply for the lamp, since the ultra-violet spectrum must be relatively intense. In the visible range, where pigments such as carotenoids and heme compounds are investigated, a tungsten ribbon light source and conventional optics may be used. These would also suffice for the near infra-red region, if desired.

Most microspectrophotometers use conventional photodetectors as the sensing element, i.e., photomultipliers for the u-v visible range. A blue sensitive tube should be used for the u-v work, of course. A possible alternative is a CdSe photoconductive cell (Clairex) which has a usable response in the u-v, peaks in the visible, and cuts off at about 900 mμ. Unfortunately, the long time constant of these photocells at low light levels makes recording difficult, although not impossible. These cells are very well suited, however, for point-by-point plotting of the data.

Another problem of microspectrophotometry is preparation of the sample to be studied. For in-vivo or in-vitro biological studies movement of the organism is hard to prevent. In addition, for the visible range, the specimen must be rather heavily pigmented, since the absorption path may be very short.

In general, two types of data may be obtained with a microspectrophotometer: (1)characteristic absorption spectra obtained by plotting optical density vs. wavelength at a particular cellular location and (2) the location and/or identification of particular sample constituents, obtained by scanning the sample at fixed wavelengths. Case (1) is com-

pletely equivalent to conventional spectrophotometry except that it is performed through a microscope. Case (2) is unique in microspectrophotemetry in that it permits one to determine whether a certain type of compound is present and where it is located within the sample. For certain compounds (nucleic acids), quantitative determinations can be made, although usually with some difficulty with regards to specimen preparation.

Recording the microspectrophotometers are relatively complicated systems. The incident light beam is chopped so that a.c. amplification may be used after the photomultiplier tube. The signal consists of the ratio of two light intensities, the sample and reference transmission. Several different types of electronic amplifiers must be used, including dynode control of the photomultiplier tube.

If photoconductive cells are used as the sensing element, some simplification of design is possible. This is the problem under study by Strother. The present aim of this work is to design and construct a recording microspectrophotometer using as many solid-state components as possible, i.e., with transistor circuitry and photoconductive cells. This type of instrument should have a high reliability and light weight, both desirable from the viewpoint of possible space exploration applications.

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