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GENETIC MECHANISMS IN BACTERIA AND BACTERIAL VIRUSES I

EXPERIMENTS ON SPONTANEOUS AND CHEMICALLY INDUCED MUTATIONS OF BACTERIA GROWING IN THE CHEMOSTAT

AARON NOVICK AND LEO SZILARD

Institute of Radiobiology and Biophysics, The University of Chicago

In an earlier paper we reported observations on the spontaneous mutations of bacteria occurring in a continuous flow device we call the Chemostat (Novick and Szilard, 1950a). In this device we maintain a bacterial population in the growth phase over an indefinite period of time by maintaining the concentration of one of the growth factors—called the controlling growth factor—at a low fixed value.

A number of different mutations will occur in a population so maintained in the Chemostat, but in the experiments reported here we are concerned only with the mutation from sensitivity to the bacterial virus T5 to resistance to this virus. In the absence of selection for or against the mutant, and if reverse mutations can be neglected, the mutant population in the Chemostat will increase linearly with time, over periods of time short enough to disregard "evolutionary" changes (Novick and Szilard, 1950a).

By plotting the number of mutants against time we obtain a straight line whose slope is proportional to the mutation rate. With tryptophane as the controlling growth factor we found for mutants resistant to T5 that the mutation rate per hour is independent of the growth rate (Novick and Szilard, 1950a) within the range of generation times, γ, between $\tau_1 = 2$ hours and $\tau_1 = 12$ hours.

In our present experiments we have used a modified model of the Chemostat, shown in Figure 1, instead of the previously described model (Novick and Szilard, 1950b). In the new model an electromagnetically operated valve, shown in the figure, is controlled by a clock and is opened for a short time at fixed time intervals. There are two pressure regulators with water levels at heights of h_3 and h_4 which can be adjusted at will. The sum of h_3 and h_4 must be so adjusted that when the valve is closed the nutrient liquid rises from the storage tank into a capillary to a height of h_1 which is too low to permit an overflow of the liquid into the growth tube. When the valve is opened a certain fraction of the liquid contained in the capillary is blown (by an air pressure whose magnitude is set by h_4) into the growth tube, while the rest of the liquid returns into the storage tank. The average flow rate of the nutrient into the growth tube can be regulated either by changing h_1 (the height to which the liquid rises in the capillary when the valve is closed) or by changing the time intervals at which the clock operates the valve.

The nutrient medium used in our experiments was a simple synthetic medium (Friedlein) containing lactate, ammonium chloride, phosphate buffer, and magnesium sulfate. Tryptophane was added when required. When we use phosphorus as the controlling growth factor, we employ tris (hydroxymethyl) amino methane in a $^1/_{50}$th molar concentration as a buffer. The B strain of *E. coli*, or mutants of this strain, was used in all of our experiments.

Spontaneous Mutations

In order to see whether the spontaneous mutation rate depends on the choice of the controlling growth factor, experiments were performed in which tryptophane, ammonium chloride (our nitrogen source), phosphate, or lactate (our carbon source) was used as the controlling growth factor. As may be seen from Figure 2, the spontaneous mutation rate is appreciably lower with ammonium chloride, phosphate, or lactate than with tryptophane as the controlling growth factor.

Earlier (Novick and Szilard, 1950a), with tryptophane as the controlling growth factor, the mutation rate was found to be about 1.25×10^{-8} per hour per bacterium. Our present, more accurate measurements indicate, however, that the real value is probably close to 1.5×10^{-8} per hour.

The mutation rate appears to be lowest when lactate is the controlling growth factor—about

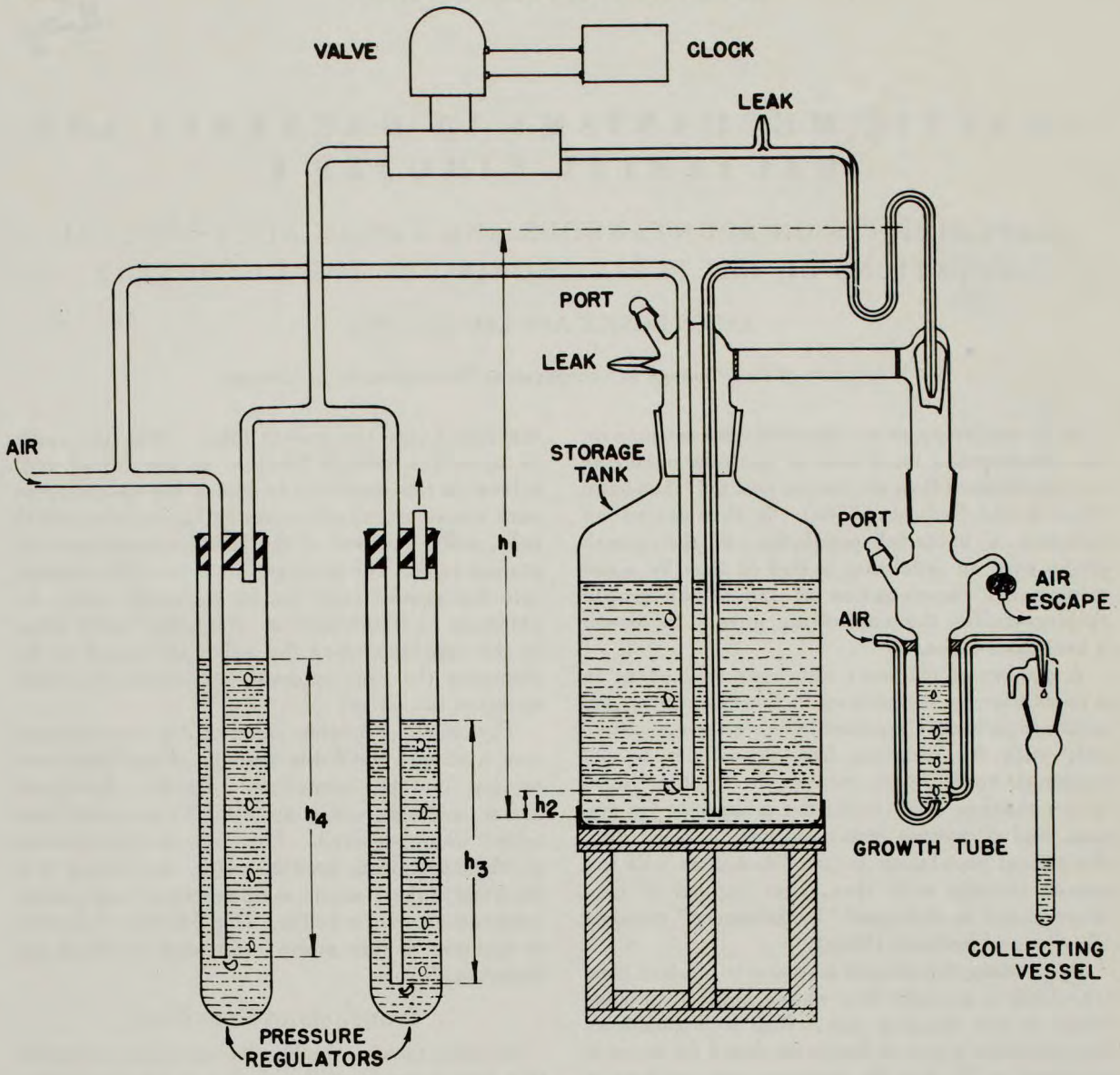

FIGURE 1.

one-third of the value obtained with tryptophane as the controlling growth factor. Because of this low value, our earlier measurements led us to believe that with lactate as the controlling growth factor the number of mutants does not increase at all with time, but stays at a constant level as the result of selection against the mutant (Novick and Szilard, 1950a). Our present findings with lactate as the controlling growth factor do not, however, indicate such selection which, if present, would lead to a curved line instead of the straight line appearing in Figure 2.

One may ask whether the high value of the spontaneous mutation rate obtained with tryptophane as the controlling growth factor is due to the specific choice of tryptophane or whether other amino acids, when chosen as the controlling growth factor, give the same spontaneous mutation rate. In order to learn something about this point we used a mutant of our strain of *coli* which requires both arginine and tryptophane as growth factors. This strain we obtained from an argininеless mutant of B, D84, (kindly supplied to us by A. Doermann) by picking a spontaneous T1-resistant mutant which requires tryptophane. This mutant strain we grew in two Chemostats. Both of these contained arginine as well as tryptophane in the nutrient storage tank, but

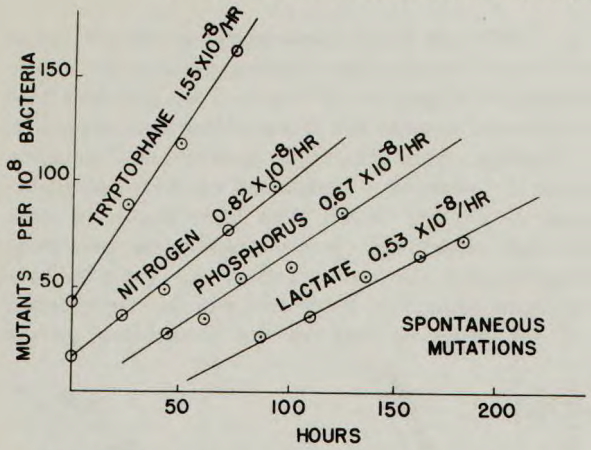

FIG. 2. Rise of spontaneously occurring T5 resistant mutants in the growth tube of the Chemostat for different controlling growth factors. For detailed data see Table 1.

one of them contained arginine in excess so that *tryptophane* was the controlling growth factor, and the other contained tryptophane in excess so that *arginine* was the controlling growth factor. As Figure 3 shows, the spontaneous mutation rate is about the same in both experiments.

If bacterial mutations arise as the result of an "error" occurring in the act of gene reproduction, one would expect the mutation rate per hour to be inversely proportional to the generation time rather than independent of it. Independence of generation time is, however, experimentally established (Novick and Szilard, 1950a) in the case of mutation to T5 resistance. In order to reconcile the error hypothesis of mutation with the experimental facts, one might say that when we slow protein synthesis by using tryptophane

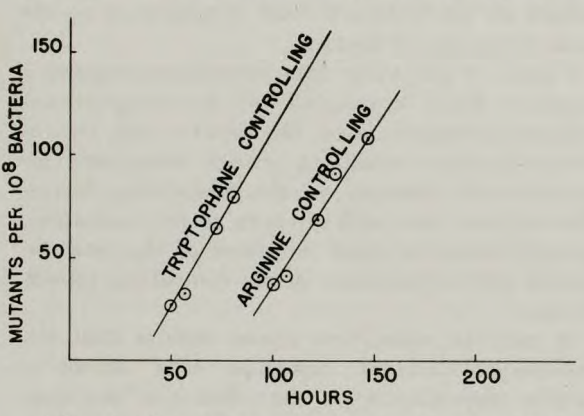

FIG. 3. Two experiments giving the rise of spontaneously occurring T5 resistant mutants with a strain requiring both tryptophane and arginine. In one experiment tryptophane was the controlling growth factor, in the other arginine. For detailed data see Table 1.

as the controlling growth factor, we might not necessarily slow the rate at which the genes reproduce; the genes might perhaps reproduce at an unchanged rate, and genes produced in excess might be discarded. Clearly, the error hypothesis of mutation demands constancy of the mutation rate per gene generation rather than bacterial generation.

Luria and Hershey (oral communications) have drawn attention to this way of possibly reconciling the observed constancy of the mutation

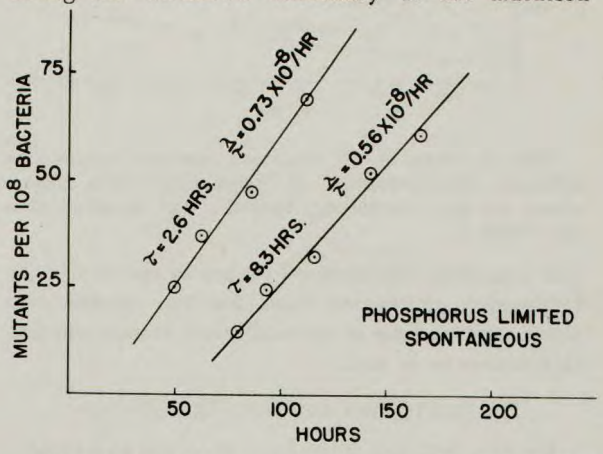

FIG. 4. Rise of spontaneously occurring T5 resistant mutants with phosphorus as the controlling growth factor for two different generation times. For detailed data see Table 1.

rate per unit time with the error hypothesis of mutation. It was further thought that if phosphorus or nitrogen were used as the controlling growth factor, the rate of gene synthesis might be slowed along with the rate of protein synthesis. Constancy of the mutation rate per gene generation demanded by the error hypothesis would then show up inasmuch as the mutation rate per hour would no longer be constant but would rather fall proportionately with increasing generation time.

In order to test this point we performed experiments with phosphorus as the controlling growth factor. In Figure 4 are represented two experiments in which the bacteria were grown with generation times of $\tau = 2.6$ hours and $\tau = 8.3$ hours. As may be seen from the figure, the mutation rate per hour is increased only slightly—if at all—by a more than three-fold increase in generation time. Thus these results do not lend any support to the error hypothesis of mutation.

With nitrogen as the controlling growth factor we again found no appreciable decrease of the mutation rate with increasing generation time.

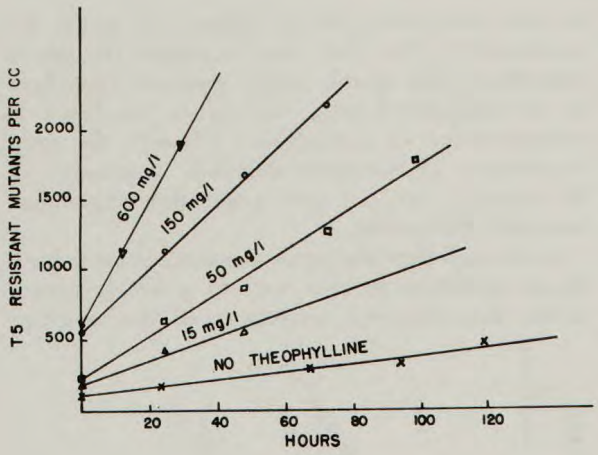

FIG. 5. Rise of T5 resistant mutants induced by different concentrations of theophylline with tryptophane as the controlling factor. For detailed data see Table 1.

The mutation rate seemed rather to increase with increasing generation time, but the accuracy of these experiments is not sufficient to say whether this increase is real.

THEOPHYLLINE-INDUCED MUTATIONS

Methylxanthines have been reported to be mutagenic for fungi (Fries and Kihlman, 1948) and also for bacteria (Bertani, Demerec and Flint, 1949). If theophylline, a dimethylxanthine, is added to the nutrient medium to bring its concentration to 150 mg/l there is no appreciable killing of the bacteria growing in the Chemostat. We find that, with tryptophane as the controlling growth factor, this concentration of theophylline raises the mutation rate from the spontaneous rate of about 1.5×10^{-8} per hour to a rate of about

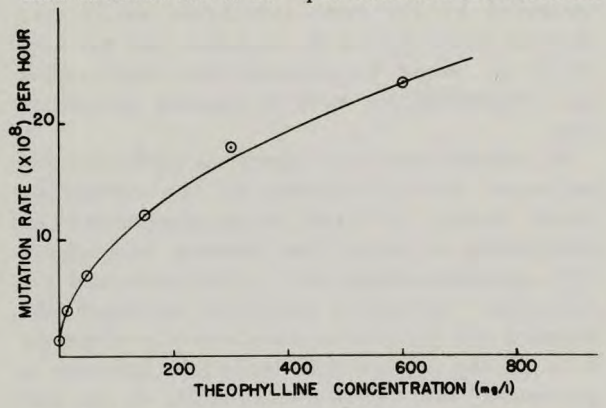

FIG. 6. Rate of theophylline-induced mutations to T5 resistance (with tryptophane as the controlling growth factor) as a function of theophylline concentration.

11×10^{-8} per hour. Measurements carried out at different theophylline concentrations are represented in Figure 5. In Figure 6 the mutation rate is plotted against the theophylline concentration.

Having found that the spontaneous mutation rate is lower when ammonium chloride, phosphorus, or lactate rather than tryptophane is used as the controlling growth factor, we performed experiments to see whether for theophylline-induced mutations there is a similar dependence of the mutation rate on the controlling growth

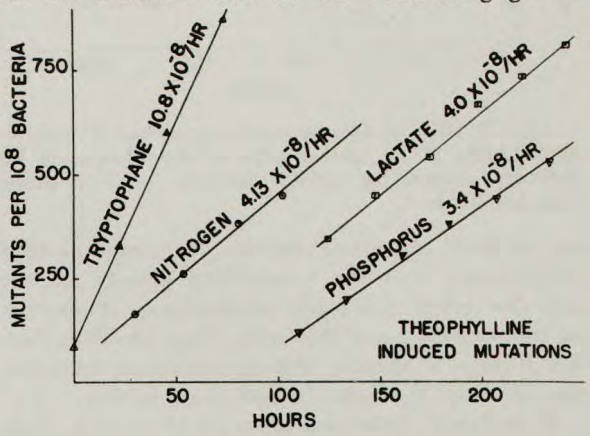

FIG. 7. Rise of theophylline-induced T5 resistant mutants in the growth tube of the Chemostat for different controlling growth factors at a theophylline concentration of 150 mg/l. For detailed data see Table 1.

factor. Figure 7 gives the results of these experiments. As the figure shows, we obtained (for the B strain) with these three controlling growth factors theophylline-induced mutation rates which are about one-third to one-half of the value obtained (for a tryptophane requiring mutant of the B strain) with tryptophane as the controlling growth factor.

Figure 8 gives for this tryptophane-requiring strain a direct comparison of the theophylline-induced mutation rates for growth with tryptophane as the controlling growth factor and for growth with nitrogen as the controlling factor. The mutation rate with nitrogen as the controlling growth factor is about one-third of the rate obtained with tryptophane as the controlling growth factor.

It may be seen from these results that the theophylline-induced mutation rate shows a similar dependence on the choice of the controlling growth factor as does the spontaneous mutation rate, being higher with tryptophane in control than with lactate, phosphorus, or nitrogen in control. This raises the question whether

TABLE 1.

	Experiment No.	Controlling growth factor	Growth factors	Strain	Bacterial density	τ hrs.	Mutagen	$\frac{\lambda}{\tau}$ (× 10^8 hr.)
Figure 2	147	Tryptophane	1000 γ/l Tryptophane	B/lt/f	4.6×10^8	4.7	None	1.55
	154	Nitrogen	30 mg/l NH_4Cl	B/r	4.0 "	4.6	"	0.82
	214	Phosphorus	3 mg/l P as phosphate	B	4.5 "	5.8	"	0.67
	222	Lactate	200 mg/l Lactic acid	B	4.6 "	4.7	"	0.53
Figure 3	232	Tryptophane	500 γ/l Tryptophane 9200 γ/l Arginine	D84/lt	3.0 "	2.4	"	1.76
	231	Arginine	2000 γ/l Tryptophane 2300 γ/l Arginine	"	2.0 "	2.6	"	1.56
Figure 4	229	Phosphorus	3 mg/l P as phosphate	B	3.7 "	2.6	"	0.73
	228	Phosphorus	" "	B	6.9 "	8.3	"	0.56
Figure 5	147	Tryptophane	1000 γ/l Tryptophane	B/lt/f	4.6 "	4.7	"	1.55
	116	"	500 γ/l Tryptophane	"	2.2 "	3.3	15 mg/l Theophylline	4.0
	117	"	" "	"	2.2 "	3.3	50 " "	7.0
	118	"	" "	"	2.2 "	3.3	150 " "	12.0
	113	"	" "	"	2.2 "	4.8	600 " "	23.8
Figure 6		Derived from Figure 5.						
Figure 7	157	Tryptophane	500 γ/l Tryptophane	B/lt	2.2 "	5.5	150 mg/l Theophylline	10.8
	201	Nitrogen	30 mg/l NH_4Cl	B	2.2 "	5.5	" "	4.13
	189	Lactate	150 mg/l Lactic acid	B	2.5 "	3.5	" "	4.0
	208	Phosphorus	3 mg/l P as phosphate	B	4.3 "	6.5	" "	3.4
Figure 8	157	Tryptophane	1 gm/l NH_4Cl 500 γ/l Tryptophane	B/lt	2.2 "	5.5	150 mg/l Theophylline	10.8
	166	Nitrogen	30 mg/l NH_4Cl 10,000 γ/l Tryptophane	B/lt	3.3 "	7.2	" "	3.5
Figure 9	225	Tryptophane	500 γ/l Tryptophane	B/lt/f	2.3 "	4.7	150 mg/l Caffeine	19.0
	157	"	" "	B/lt	2.2 "	5.5	" Theophylline	10.8
	199	"	" "	B/lt/f	2.5 "	6.2	" Paraxanthine	8.4
	226	"	" "	B/lt/f	2.3 "	4.1	" Theobromine	7.5
	184	"	" "	B/lt/f	2.5 "	4.8	" 8-Azaguanine	3.4
Figure 10	114	Tryptophane	500 γ/l Tryptophane	B/lt/f	2.2 "	6.0	500 mg/l Adenine	5.2
	124	"	" "	"	2.2 "	5.3	164 mg/l Adenine	2.95

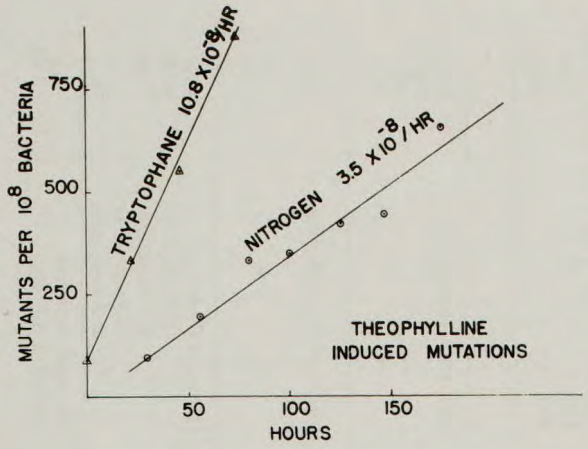

FIG. 8. Two experiments on theophylline-induced mutations to T5 resistance in a tryptophane requiring strain, B/lt, one with tryptophane, the other with nitrogen as the controlling growth factor. For detailed data see Table 1.

the processes leading to spontaneously occurring mutations have some step in common with those involved in the induction of mutations by theophylline.

Purine Derivatives other than Theophylline

With tryptophane as the controlling growth factor, caffeine, paraxanthine, theobromine, and 8-azaguanine in concentrations of 150 mg/l have, like theophylline, a marked effect on the mutation rate. This may be seen from the data shown in Figure 9.

The effect of adenine on the mutation rate—with tryptophane as the controlling growth factor—

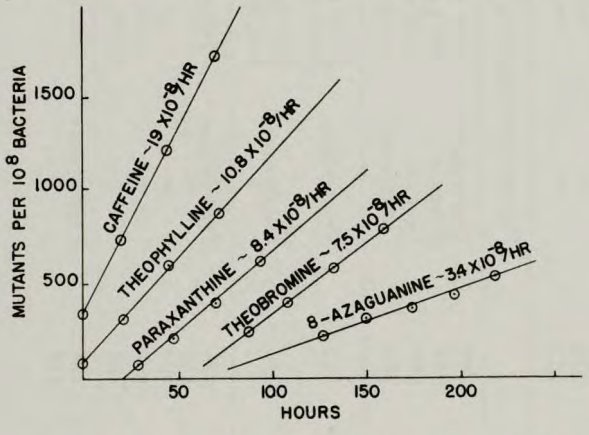

FIG. 9. Mutations to T5 resistance induced by a number of purine derivatives at concentrations of 150 mg/l with tryptophane as the controlling growth factor. For detailed data see Table 1.

was determined for two different concentrations and is shown in Figure 10.

2—6, diamino purine in a concentration of 150 mg/l did not appear to be mutagenic, at least not within the limits of our experimental error.

Of the purine derivatives which we found to be mutagenic, caffeine, theophylline, paraxanthine, and theobromine are all N-methyl xanthines. Because purines are constituents of nucleic acid and nucleic acid is a constituent of the cell

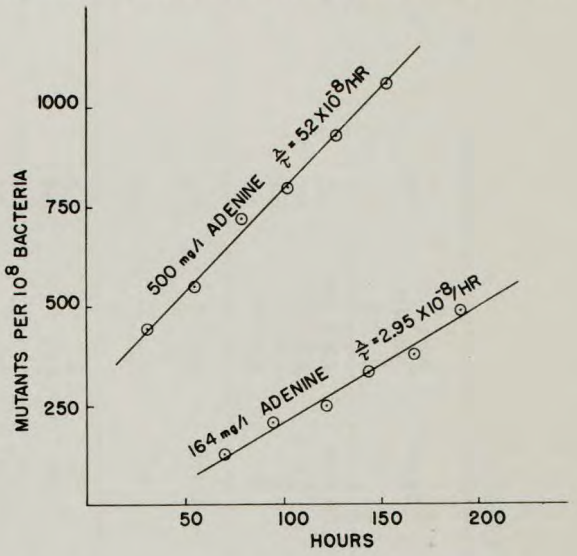

FIG. 10. Mutations to T5 resistance induced by two different concentrations of adenine with tryptophane as the controlling growth factor.

nucleus, one might think that the likeness of the mutagenic purine derivatives to the naturally occurring purines has something to do with their mutagenic action.

If this were so, one might also expect some pyrimidine derivatives to be mutagenic. We therefore tested for mutagenic action, using tryptophane as the controlling growth factor, the following pyrimidine derivatives: uracil, thymine, 6-methyl uracil, 5-amino uracil, 5-bromo uracil. None of these appeared to be mutagenic in a concentration of 150 mg/l. Experiments with other pyrimidine derivatives are in progress.

The authors wish to express their gratitude to Dr. George H. Hitchings of the Wellcome Research Laboratories, who very kindly put at their disposal many of the purine and pyrimidine derivatives used in these experiments. The authors gratefully acknowledge the support of this work by a grant from the National Institutes of Health of the United States Public Health Service.

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DISCUSSION

MARSHAK: Dr. Novick has pointed out that there is no correlation in his experiments between the mutagenicity of a compound and its presence as a constituent of nucleic acid. In this case presence or absence does not appear to be an adequate basis of comparison. Actually on the basis of tracer studies there is a very good correlation between the observations that adenine is readily incorporated into nucleic acid while the pyrimidines are not with his finding that adenine and some of its derivatives are mutagenic while the pyrimidines are not. Another parallelism may be found with the results of analysis of the composition of nucleic acids. I have found that the nuclear pentosenucleic acids of several different tissues in the same animal all have approximately the same proportions of the purines adenine and guanine, but the pyrimidine ratios vary widely.

SZYBALSKI: The idea of the "Chemostat," that is, the idea of continuous propagation of microorganisms (used often in fermentation industries) controlled by a growth limiting factor (described also in a paper of J. Monod, 1950, Inst. Pasteur *79*: 390), is obviously a very ingenious tool for studying microbial genetics.

It has however, some disadvantages since in some cases it is difficult to be certain that the use of marked, deficient strains or "one-factor starvation" has no influence on the primary effect under investigation.

It seems to me that a "Turbidostat" (if I can introduce this name) which makes use of the turbidity of the culture to control and limit growth is a more general and perhaps even simpler solution of this problem. Such a device based on the principle of photoelectric control is now being used in our laboratory for studying mutations (V. Bryson).

NOVICK: One of the characteristic features of the Chemostat is the independence of the concentration of the controlling growth factor in the growth tube in the stationary state from the concentration of this factor in the incoming nutrient. It is on account of this feature that we called the device "Chemostat." We did not recognize this feature until the summer of 1948 even though we discussed building such a device for the study of bacterial adaptations while we were working at Cold Spring Harbor in the summer of 1947. We have been working with the Chemostat since October 1948, and find it capable of a much greater variety of applications than the "Turbidostat." The possibility of varying the generation time and also the controlling growth factor gives it a greater flexibility. We do not consider "one-factor starvation" a disadvantage provided we know what the factor is and can change it at will.