THE REVERSIBLE SUPPRESSION OF STATIONARY PHASE MUTATION IN *ESCHERICHIA COLI* BY CAFFEINE

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BACTERIAL growth ceases when an essential constituent is exhausted. The bacteria show no further increase in mass, in viable cell number, or after a short time, in total amount of DNA. The proportion of viable cells in the population may decrease at a constant rate, but no measurable decrease in total cell mass or number is detectable for the first 300 hours (RYAN 1959). The cells are said to be in stationary phase (S.P.). Such a state can be induced in auxotrophic bacteria by limiting the required growth factor.

When cultures of a histidine requiring (*his*$^-$) strain of *Escherichia coli* 15 ceased growing due to the exhaustion of histidine in the medium, RYAN noted that mutations from auxotrophy to prototrophy continued to occur, although at a somewhat reduced rate. The mutation rate μ during growth was 3×10^{-8} per cell per hour (when the generation time was 50 min); during the stationary phase it was 1×10^{-9} per cell per hour (RYAN 1959). The S.P. mutation rate remained constant even when the *his*$^-$ bacteria were dying exponentially, provided the culture conditions were not altered.

RYAN (1957) satisfactorily demonstrated that these prototrophic clones arose during the S.P., and were not *his*$^+$ cells starting to grow after an exceptionally long lag or at an abnormally slow rate. In a series of careful experiments he also showed that little or no cell turnover occurred (RYAN 1957, 1959). Given the probability of mutation of about 3×10^{-8} per generation, one would have to postulate about 2% cell turnover per hour to account for the observed rate of mutation during the stationary phase. Since the tests covered periods of 200 to 400 hours, one tenth of this rate would have been readily detected. Although turnover of whole cells during stationary phase appeared unlikely as an explanation of S.P. mutation, cryptic turnover of nucleotides in DNA and an associated probability of mutation was not eliminated as a causal factor.

ZAMENHOF, RICH and DEGIOVANNI (1959) found that incorporation of 5-bromouracil into DNA of a growing culture of *E. coli* continued into stationary phase. Moreover, uptake of C^{14} thymidine in stationary-phase strain 15 *his*$^-$ bacteria has been observed (RYAN, personal communication). In the absence of any net increase in the total amount of DNA this must mean that nucleotides were being replaced continuously.

An enzymatic process leading to the replacement of nucleotides in DNA has been discovered recently during studies of the mechanism by which cells recover

TABLE 1

Effect of caffeine on survival of ultraviolet irradiated his⁻ E. coli *15 bacteria*

Period after irradiation before bacteria were spread on nutrient medium	Percent surviving		
	Control	.004 M Caffeine	.008 M Caffeine
0	2.45%	0.087%	0.028%
2 hours	12.83%	1.12%	0.41%

Unirradiated and UV irradiated bacteria from an overnight sugar-limited stationary phase culture, washed and irradiated in saline were diluted in saline and spread on broth agar and broth agar supplemented with .004 M and .008 M caffeine, both immediately and after incubation in the dark for 2 rours at 37°.

from irradiation damage. So called "dark-repair" of genetic lesions caused by ultraviolet light (UV) is thought to involve excision of abnormal oligonucleotides by an endonuclease, followed by repair of the gap in the nucleotide chain by polymerisation of new nucleotides perhaps in a sequence determined by the exposed section of the complementary strand (SETLOW and CARRIER 1964; BOYCE and HOWARD-FLANDERS 1964). If this nucleotide excision-replacement system functions in the unirradiated cell, albeit at a lower rate, to carry out normal DNA repair and maintenance, it could well be the way in which nucleotide "turnover" as deduced by uptake of labelled precursors, occurs.

Dark-repair or the related host-cell reactivation is affected by a number of extrinsic and intrinsic factors (WITKIN 1959; LIEB 1964; HARM 1963). Post-irradiation treatment with caffeine inhibits it. There is a suggestion that this occurs by specific inhibition of the enzymes involved (SAUERBIER 1964). That caffeine inhibits dark repair in *E. coli* 15 *his*⁻ cells was verified experimentally (see Table 1).

If nucleotide turnover associated with the dark repair system is the basis of S.P. mutation, inhibition of one or other of the enzymatic steps involved should decrease the rate of S.P. mutation. We have attempted to test this idea. Caffeine, which itself is slightly mutagenic, but only to cells permitted to replicate (GLASS and NOVICK 1959), was used as the inhibitor of the dark repair process.

We will demonstrate that this purine is a potent suppressor of stationary phase mutation but is without antimutagenic effect of replication mutation.

MATERIALS AND METHODS

Stock cultures: The *his*⁻ mutant was kindly provided by PROFFESSOR F. J. RYAN. It was a subculture of the stock used by him in earlier studies of S.P. mutation (RYAN 1955). The *his*+ stock used in tests of the experimental method was derived by S.P. mutation from *his*⁻.

Basal medium: A modified Gray and Tatum recipe (RYAN 1959). When used as a growth medium either .05% or .1% glucose was added; when used as a maintenance medium for stationary phase cultures it contained either 0.5% glucose or 0.5% lactose.

Histidine growth medium: Basal medium supplemented with 30μg/ml of L-histidine.

Broth agar medium: Oxoid "Blood Agar Base."

Caffeine: Either B.P. grade (Evans, Liverpool) or a sixfold recrystallised sample derived from the B.P. grade product. Each was kept as a 2% sterile solution and used to supplement media.

All concentrations of solutes are expressed as w/v.

Mutation assay: The rate of mutation from auxotrophy to prototrophy was measured in a histidine requiring mutant (*his*⁻) of *E. coli* 15 maintained on a selective medium lacking histidine. Cultures were grown overnight in a histidine medum until the glucose was exhausted. The bacteria were washed twice and quantities of cells measured onto or into the appropriate test media. The viabilities of the *his*⁻ bacteria in the several different treatment groups were determined daily during the course of each experiment by spreading cell suspensions suitably diluted in 0.9% saline on broth agar medium. In the tube culture experiments, estimates of viabilities were based on determinations on two or more replicate cultures per treatment group. In the agar plate and membrane culture experiments, the *his*⁻ population was washed off a different membrane culture each day after all visible *his*+ colonies had been excised, diluted suitably and spread on broth agar plates. Colonies were counted after 24 hours incubation at 37°.

Membrane culture experiments: Disks of dialysis membrane 7.5 cm in diameter were placed on basal agar plates containing 30 ml of medium. Bubbles beneath the membranes were expressed with a glass rod spreader. The membranes were covered with a film of basal medium by pouring a few ml of hot medium over the surface and immediately pouring off the excess. Suspensions of between 5×10^7 and 3×10^8 bacteria were spread over the membrane agar surface in the usual fashion and incubated at 37° for one day on basal medium to allow the background *his*+ cells to form macroscopic colonies. Half of the cultures were transferred to fresh basal medium and half to caffeine medium (0.004 M or 0.008 M). *his*+ colonies present were scored and colour-marked. All cultures were transferred to fresh medium 2 days later and after a further 2 days both groups of membrane cultures were transferred to fresh basal medium. Newly arisen colonies were marked and scored daily.

Tube culture experiments: Basal medium containing 0.5% glucose and supplemented with sufficient histidine (.025 μg/ml) to allow the growth of 5×10^6 *his*⁻ bacteria per ml was inoculated with a small number of *his*⁻ cells. Two-ml quantities were dispensed into 400 to 500 $6 \times \frac{1}{2}$ inch test tubes. The S.P. cultures were incubated 3 days at 25° to allow all background *his*+ bacteria to grow to turbid cultures (the average time taken for one *his*+ bacterium to grow to 10^8 cells was 52 hours under our culture conditions) and the turbid cultures were removed from the population. The clear cultures which contained only *his*⁻ bacteria were supplemented with either caffeine (final concentration .008 M) or an equal volume of distilled water, and reincubated. After a period of incubation during which turbid tubes were scored daily, 10 ml of basal medium was added to each of the remaining clear tube suspensions in both control and caffeine treated groups, i.e. the original cultures were diluted sixfold, incubated further at 25° and turbid tubes which arose were scored as before. Immediately prior to dilution a number of clear-tube cultures were withdrawn from both control and caffeine groups and a few (an average of 3.5) *his*+ bacteria inoculated into each of them. These were incubated at 25° and the period required for each tube to become turbid was measured.

In some experiments glucose was replaced as an energy source by lactose. The 15 *his*⁻ strain is *lac*+ (*i*+,*y*+,*z*+) and, provided the lactose is added to previously glucose grown bacteria which are amino-acid starved, beta-galactosidase synthesis remains repressed, the sugar is split only slowly (at about 1% of the derepressed rate) and no measurable death occurs for more than 300 hours (RYAN 1959). S.P. mutation occurs, but at a reduced rate. In lactose experiments, the *his*⁻ cultures were grown as in the glucose ones but sufficient glucose (10 μg/ml) was added to the medium so that a small excess was present when the histidine was exhausted. Subsequently sufficient lactose was added to make the final concentration 0.5% (for details see RYAN 1959). Following the expression and removal of background *his*+ bacteria, the caffeine solution was added and the experiment proceeded as outlined above.

General: All colonies and turbid cultures were streaked out on basal and histidine media. Only cultures which had normal *his*+ growth characteristics were scored as *his*+ and used for estimating the S.P. mutation rate.

The mean number of *his*+ back-mutants per tube culture m_t was estimated from $P_0(t) = e^{-m_t}$ each day where $P_0(t)$ is the proportion of nonturbid tubes (RYAN 1959) at time t. The mean number of reversions per *his*⁻ bacteria per 24 hours $= m_t/v_{t-a}$ where v_{t-a} is the viability "a"

hours before the observation of m_t was made at time t hours. Since cultures were scored only once a day, viabilities were obtained from curves of viable cell number plotted against time. In determining v_{t-a}, a was taken as 24 hours for the membrane culture experiments at 37° and 52 hours for the tube experiments at 25°, the respective times necessary for a *his*+ bacterium to grow to a macroscopic colony at 37° or a turbid culture at 25°. μ, the mutation rate per hour, was obtained from the slope of the frequency of mutants plotted against time.

Effect of caffeine on glucose uptake: Stationary phase cultures of *his*⁻ bacteria in basal medium containing 0.5% glucose were shaken continuously at 37°. A quantity of concentrated caffeine solution or a similar volume of water was added to the flasks to make the final concentrations 0, .004 M, and .008 M. Samples were taken periodically and their glucose content determined by a specific glucose oxidase method using the Glucostat reagent of Worthington Biochemical Corp.

RESULTS

Stationary phase mutation: During growth of the *his*⁻ cultures, prototrophs arose with a frequency of about 3×10^{-8} per cell generation (cf. RYAN 1959). When growth ceased with exhaustion of histidine, *his*+ mutants continued to appear. Initially the rate of appearance of these clones was relatively high as preexisting or "background" *his*+ cells overgrew the *his*⁻ cultures, and this is shown in Figures 1, 2 and 3 as a sharp rise in the curve relating the mean frequency of mutants per culture (m) and time. Subsequently, in all experiments the mutation rate (μ) declined to another relatively constant value, the stationary phase mutation rate (see also RYAN 1955, 1959). In the ten S.P. mutation experiments which we performed, *his*⁻ cultures were incubated at 25° or 37° on solid or liquid medium containing either glucose or lactose. The mutation rate and the rate of death varied with each combination of environmental factors chosen but

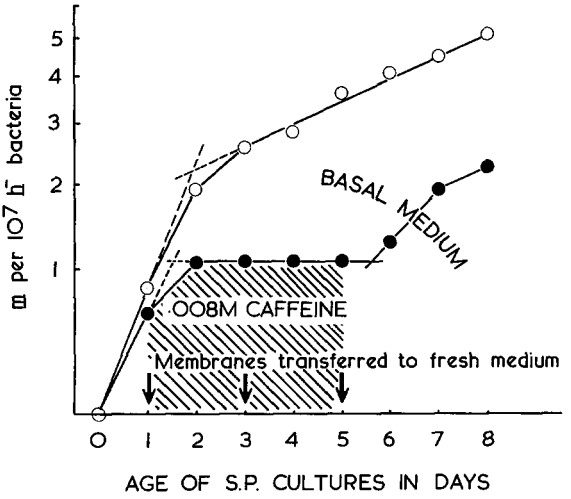

FIGURE 1.—Reversible inhibition of *his*⁻ → *his*+ mutations by caffeine during the stationary phase: membrane cultures. Incubation temperature, 37°C. The data are based on 33 replicate cultures of 10^8 *his*⁻ cells in each group. O—Control; ●—Caffeine treated. m is the average number of *his*+ colonies per membrane. The mutation rate μ may be obtained from the slopes of the curves.

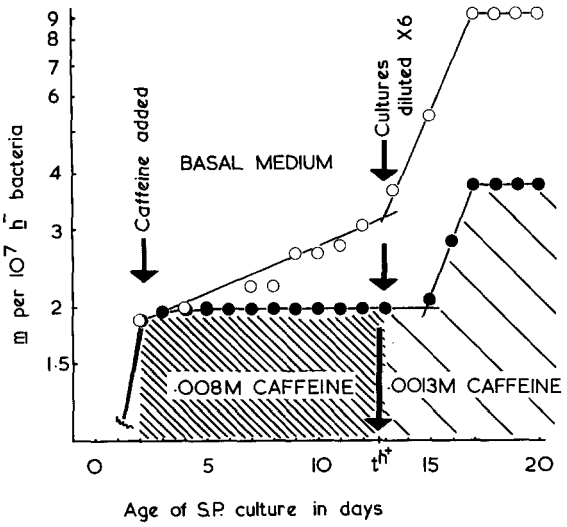

FIGURE 2.—Reversible inhibition of *his*⁻ → *his*+ mutations by caffeine during the stationary phase: liquid glucose medium. The *his*⁻ cells died at the rat eof 0.4% per hr. The graph is truncated at 16 days when the number of bacteria had reached such low levels that reliable estimates of *m* could not be made. *m* is the average number of *his*+ mutants per tube culture estimated by the proportion of nonturbid tubes at any particular time. O—Control; ●—Caffeine treated.

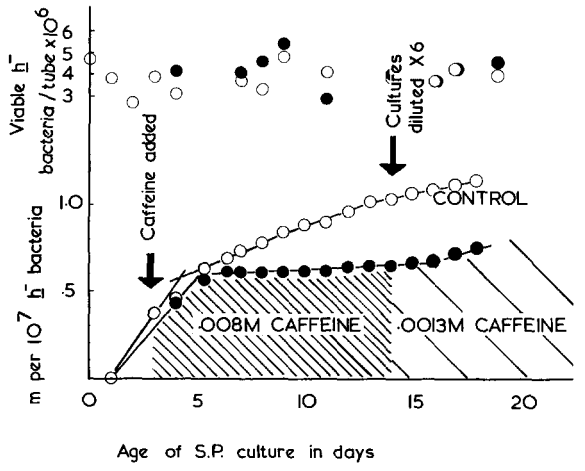

FIGURE 3.—Reversible inhibition of *his* → *his*+ mutation by caffeine during stationary phase: liquid lactose medium. Incubation temperature, 25°. The viable number of the nonadapted (lac+) *his*⁻ bacteria (4×10^6 per culture) did not change during the mutation experiment. O—Control; ●—Caffeine treated. For definitions of symbols see Figures 1 and 2.

the form of the mutation curve was similar in each. No loss of viability occurred in nonadapted *his*⁻ cells in lactose liquid medium during the experiments (Figure 3). However, bacteria which had adapted to lactose died at the same rate as in glucose medium, namely 0.4% per hour at 25°C. The rates of death on agar or

membrane culture often varied during experiments, but in the experiment illustrated in Figure 1, there was no reduction of viability during the experiment.

On membane cultures at 37° transferred to fresh medium each two days, the s.p. mutation rate, μ was 2.3×10^{-9}/hr. In liquid cultures at 25° the rate was rather less at 0.5×10^{-9}/hr for glucose medium and adapted bacteria on lactose medium and 0.2×10^{-9} for nonadapted cells on lactose medium.

When caffeine (.008 M = .15%) was added to the cultures, the s p. mutation rate fell. The effect of the drug was immediate (Figures 1, 2, 3) when due allowance is made for the lag between the formation of a *his*$^+$ cell by mutation and its growth to a macroscopic clone. Thus, following the addition of caffeine, few new *his*$^+$ colonies or turbid cultures were observed after the 24- or 52-hour period required for preexisting *his*$^+$ bacteria in the population to be scored on plate or in tube cultures respectively. .004 M caffeine, though less effective, still significantly reduced the s.p. mutation rate. In no experiment did either level of caffeine alter the rate of death of the cells during the stationary phase.

The *his*$^+$ bacteria, inoculated into a random sample of 12 clear-tube cultures immediately prior to dilution of the latter with fresh medium (at time t_{h^+}) to test their ability to support growth of revertants, grew to turbid cultures within 60 hours in both control and caffeine groups in each of the liquid culture experiments.

Reversibility of the antimutagenic activity of caffeine: With the reduction of concentration of caffeine to $\leqslant$.0013 M by either diluting the liquid caffeine medium sixfold with basal medium or by transferring membranes from caffeine to basal medium, *his*$^+$ clones began to appear again 52 or 24 hours later (Figures 1, 2, 3). In the liquid cultures, the mutation rate increased to that of the controls. On the agar medium, it may even seem to be higher than the control, but because of the difficulties of accurately determining viabilities of the *his*$^-$ cells on membrane cultures we do not ascribe any significance to this. In tube experiments with glucose medium (Figure 2), dilution of cultures containing $\geqslant 5 \times 10^6$ *his*$^-$ cells/ml appeared to increase the mutation rate in both caffeine and control groups, but we do not wish to stress the quantitative significance of this increase rate. A systematic underestimate of viable cell number and a consequent overestimate of μ could have occurred following dilution of the tube cultures since stirring the almost filled tubes was difficult and possibly inefficient. Subsequently, care was taken to eliminate such a source of error. In experiments on membrane cultures, we noted that there seemed to be some inverse relation between density of *his*$^-$ bacteria per plate, membrane, or liquid cultures and μ (Grigg and Stuckey, unpublished) so the enhancement of mutation rate with dilution was not unexpected. Had we not direct experimental evidence immediately before dilution that both control and caffeine cultures supported normal growth of *his*$^+$ bacteria inoculated into them (see above), we might have suspected the presence of cryptic *his*$^+$ bacteria in both control and caffeine cultures which were expressed upon subsequent dilution.

Whether or not the rates of s.p. mutation following dilution of the cultures were systematically overestimated in both control and caffeine groups in one

experiment, the fact remains that S.P. mutation is arrested by the addition of .008 M caffeine but this inhibition is released when the concentration is lowered to .0013 M. As with its inhibitory effect on dark reactivation (SAUERBIER 1964) the S.P. antimutagenic activity of caffeine seems to be reversible.

Reconstruction experiments to test the effect of caffeine and his⁻ *populations on the growth of single* his⁺ *bacteria.* Even though the similarity in numbers of *his*+ revertants which appeared one day after transferring the cultures to caffeine or control medium at 37° (Figure 1) did not suggest any interference of *his*⁻ cells or caffeine with the growth of *his*+ bacteria, an interaction of the two agents has not been formally excluded. While singly ineffective their combination might lead to "Competitive Suppression" (GRIGG 1958, 1964) and thus bias the results.

These possibilities were tested by examining the effect of caffeine, with and without added *his*⁻ populations on the growth of *his*+ bacteria.

In some experiments, equal numbers of *his*+ bacteria (about 70 per plate) were spread on membrane agar plates either supplemented or nonsupplemented with .008 M caffeine. In others 2×10^8 *his*⁻ bacteria were added with the *his*+ bacteria to the two types of medium used. The *his*⁻ populations contained numbers of cryptic background *his*+ reversions which gave rise to about 35 *his*+ colonies per plate.

Although on the caffeine medium the appearance of the *his*+ colonies was delayed slightly when compared to the controls, the total number of prototrophic colonies on the plates spread with the artificial mixture of *his*⁻ + *his*+ bacteria did not differ significantly between the two media (Control : 11.4 ± 6.1, Caffeine : 101.8 ± 5.7) (Table 3). Thus under conditions approximating those in the S.P. experiments, there is no evidence of a significantly large selective suppression of *his*+ bacteria by caffeine medium or of competitive suppression by the *his*⁻ bacterial cell concentration used on the control plates. On the other hand, the chance of *his*+ bacteria forming colonies was lower on caffeine than on control medium (e.g. in the plating experiment reported in Table 3 the number of colonies on caffeine plates was 59.0 ± 5.4; on controls 86.8 ± 7.5). This deficiency, which has been confirmed in other experiments, appears to be due to the induction of 0.3 "lethal events" per cell replication at 37°. At 25° the frequency of these events is lower (GRIGG, unpublished).

Effect of caffeine on replication-mutation: Caffeine is known to be mutagenic to bacteria during growth (GLASS and NOVICK 1959). Nevertheless we thought it worthwhile to check that it had no inhibitory effect on replication mutation. Should it fail to inhibit the production or expression of *his*+ revertants during the growth of a *his*⁻ culture we could conclude that it was unlikely that caffeine suppressed S.P. mutation by inhibiting the expression of cryptic *his*+ revertants.

A suspension of about 10^6 *his*⁻ bacteria in 2 ml of liquid medium was pipetted into each of 440 test tubes (6 × ½ inch). The medium contained sufficient histidine (0.05μg/ml) to allow a 30-fold increase in cell number and an excess of glucose (.5%). Half of the tubes were supplemented with caffeine (.008 M) and half were supplemented with an equal volume (0.1 ml) of water. During growth a number of *his*+ revertants were produced which gave rise to turbid cultures. From the frequency, P_0, of nonturbid cultures the mean number of *his*+ reversions per tube culture m was estimated from the expression $P_0 = e^{-m}$. The mutation rate u can be obtained from $\mu = m\ (\ln 2/N)$ (N being the number of viable *his*⁻ cells per tube when growth ceased with the exhaustion of histidine in the medium).

In deriving this expression for μ the number of cell replications during growth is taken as $\simeq$ N. This approximation is very close when a culture is grown from a small inoculum. In the

TABLE 2

The effect of caffeine on replication mutation

A. *Increase in cell number during growth*

	Number of viable cells per tube culture*	
	Basal medium	Caffeine supplemented medium
Initial inoculum	$1.4 \pm 0.047 \times 10^6$	$1.4 \pm 0.047 \times 10^6$
At cessation of growth	$3.32 \pm 0.45 \times 10^7$†	$1.7 \pm 0.07 \times 10^7$†

B. *Number of mutations which occurred during growth and estimates of mutation rates*

Period in s.p. (25°) before turbid cultures scored	Basal medium			Caffeine supplemented medium		
	P_0	m^1	$\mu \times 10^8$	P_0	m^1	$\mu \times 10^8$
23 hr	1	...	...	1	...	...
50 hr	0.202	1.60	*3.34*	0.581	0.54	2.24
70 hr	0.161	1.83	3.83	0.324	1.13	*4.66*
89 hr	0.138	1.98	4.13	0.301	1.20	4.95

P_0 is the proportion of nonturbid cultures, m^1 the mean number of revertants per culture, and μ the mutation rate/bacterium/generation.

* Based on measurements on three different cultures in each group.

† *Total* cell count identical in both groups although the *viable* cell count differed.

experiment considered here where only a 30-fold increase in cell number occurred during growth the number of replications will be overestimated by about 3%—a trivial error which can be ignored.

The results of one such experiment in which replication mutation was measured during a 20-fold increase in cell number at 25° are shown in Table 2 and indicate that caffeine does not decrease reverse mutation of *his*$^-$ → *his*$^+$ during growth. *his*$^+$ cultures grew a little slower on .008 M caffeine medium than on the control medium and estimates of mutation rate based on the frequency of nonturbid cultures in the control group at 50 hours are best compared with that of the caffeine group at 70 hours when the next observations were made. Whichever way the comparisons are made, however, qualitatively similar conclusions result. RYAN (1959) had measured μ to be about 3×10^{-8}/bacterium/generation. In our experiments $\mu = 3.34 \times 10^{-8}$ in the control groups (based on P_0 scored at 50 hours after growth ceased. This is not biased by the inclusion of stationary phase mutations since it takes about 50 hours for 1 *his*$^+$ bacterium to grow to 5×10^7/ml and render a culture turbid). In the caffeine group $\mu = 4.66 \times 10^{-8}$ (or 2.24 if P_0 scored at 50 hours is used in estimating μ). The quantitative agreement between replication mutation rates in control and caffeine groups is thus quite close. If anything, μ is higher in the caffeine group—a not unexpected result because of the known mutagenic effect of caffeine to growing bacteria. Similar results were obtained in mutation experiments on agar medium. Although the viability of *his*$^-$ bacteria grown on caffeine containing medium was lower than on basal medium the total cell population was identical on the two media. The significance of this observation will be discussed in a later report.

Is caffeine the active agent? Since commercial grades of caffeine contain a number of minor contaminants one of these rather than caffeine might be the

active substance. We compared the biological activity of crude (B.P. grade) caffeine and a purified six-times recrystallised sample of caffeine using the following criteria: (1) Inhibition of dark repair of UV induced damage; (2) production of "dominant lethals events" in unirradiated *his*$^+$ and *his*$^-$ bacteria during growth on caffeine medium, and (3) inhibition of S.P. mutation. By all three criteria the two caffeine samples had identical specific activity. Of the three, perhaps the first is the most sensitive since halving the concentration of the B.P. grade caffeine from .008 M to .004 M increased the survival of irradiated cells threefold (see Table 1). The survival of UV irradiated bacteria on broth agar alone and broth agar supplemented with either .004 M caffenie (B.P. grade) or .004 M caffeine (recryst. 6×) was respectively 24.3 ± 4.3%, 3.73 ± 0.7%, and 4.45 ± 0.9%; i.e. the two caffeine samples had indistinguishable specific activity. Furthermore, each induced a similar frequency of dominant lethals per replication, namely 30%, and there was no detectable difference in their antimutagenic activity as reference to Figures 2 and 3 will indicate. B.P. grade caffeine was used in the mutation experiment illustrated in Figure 2 and recrystallised caffeine in the experiment shown in Figure 3. We conclude, therefore, that caffeine was the active agent.

Caffeine and glucose uptake in stationary phase: Availability of a utilisable energy source is an important factor governing S.P. mutation rate (RYAN 1959). A depression of sugar uptake by caffeine could explain its effect on S.P. mutation. A comparison of glucose uptake in caffeine and non-caffeine treated bacteria in "shake" cultures in liquid basal medium at 37° indicated a stimulation of glucose uptake by caffeine (Figure 4) rather than a depression. The rate of uptake was 0.016 mg/10^8 bacteria/hr for the controls and 0.023 mg/10^8 bacteria/hr for cultures containing either .004 M or .008 M caffeine.

DISCUSSSION

The results indicate that caffeine lowers the rate of *his*$^-$ to *his*$^+$ mutation during stationary phase without interfering with mutation during replication and without affecting the rate of death in S.P. culture. The inhibition of S.P. mutation in

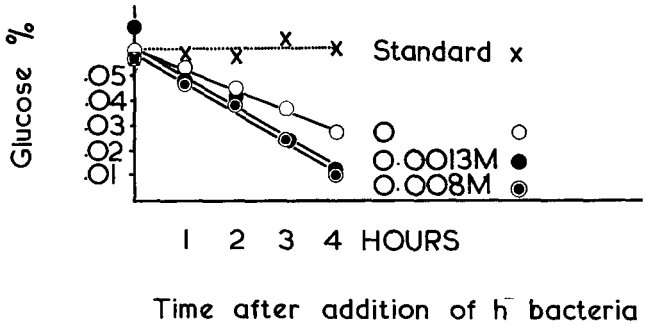

FIGURE 4.—Glucose uptake in shake cultures of strain 15 *his*$^-$ bacteria starved of histidine and containing either 0, .004 M or .008 M caffeine.

TABLE 3

Reconstruction experiments to test the effects of his⁻ *populations on the growth of small numbers of* his⁺ *cells*

Medium	Bacteria spread		
	his⁺	*his*⁻	*his*⁺ + *his*⁻
Basal	86.8 ± 7.5	39.6 ± 2.4	114.4 ± 6.1
Caffeine (.008 M), Basal	59.0 ± 5.4	33.3 ± 3.0	101.8 ± 5.7

The average number is given of *his*⁺ colonies on membrane cultures spread with either *his*⁺ bacteria or mixtures of *his*⁺ and *his*⁻ cells and incubated on the media indicated.

his⁻ bacteria by .008 M or .004 M caffeine was immediately relieved by diluting the caffeine to a concentration of ⩽.0013 M.

Under the same conditions added *his*⁺ cells or revertants which occurred during replication of the *his*⁻ population in caffeine grew to macroscopic clones, and this bars the possibility of accounting for the results by some direct or indirect suppression of growth of *his*⁺ back-mutants by caffeine. Depression of bacterial metabolism seems an equally unlikely explanation since caffeine actually increased glucose uptake during stationary phase rather than decreased it and had little adverse effect on the replication time of the cells.

.008 *M* caffeine causes 0.25 "lethal events" per replication at 25° and 0.3 at 37° in strain 15 *his*⁻ bacteria (Table 3 and GRIGG, unpublished) and we should note that the value of *m* based on the observed number of turbid cultures will be underestimated and must be corrected by these amounts. An increase in *m* of 25% or 30% during stationary phase in caffeine is, however, so small that it scarcely registers on the scale used in Figures 1, 2 and 3.

The main interest of these experiments centres on the specificity of the antimutagenic effect and the ease with which the inhibition of S.P. mutation is released by dilution of the caffeine. Presumably the same basic genetic change occurred in mutation of *his*⁻ → *his*⁺ during replication as during stationary phase, yet caffeine largely prevented the latter without interfering with the former. This suggests to us that caffeine does not directly interfere with expression of the genetic code but rather with some mechanism which, particularly during the stationary phase, is capable of altering the sequence of nucleotides.

The easy reversibility of the caffeine effect suggests that the binding between caffeine, and the reactants in the pathway leading to S.P. mutation may not be great or that caffeine is inhibiting a multitarget reaction, i.e. that there are a number of excision-repair enzymes active at any point in time. Caffeine, together with the other methyl purines, strongly inhibits the enzymatic dark repair of UV irradiation damage to DNA. This inhibition also is readily reversed by moderate dilution of the caffeine solution and led SAUERBIER (1964) to suggest that since it does not inhibit photoreactivation, caffeine suppresses the dark repair reaction by specific enzymic inhibition rather than by combination with the substrate

(DNA). It is not known which of the several steps postulated in dark repair is inhibited by caffeine. It is perhaps pertinent that the concentrations of caffeine effective as inhibitors of purine phosphorylases (KOCH and LAMOND 1956), dark repair and s. p. mutation are similar.

Although there are some data to suggest that nucleotides turnover occurs during stationary phase in *E. coli* 15 *his*$^-$ (RYAN, personal communication) there is, as yet, no definitive evidence linkage normal turnover and the caffeine-inhibited nucleotide-replacement process associated with dark repair. Our assumption that there was such a link and which leads us to this study of s.p. mutation seems plausible. We are subjecting it to experimental test.

The few reports of substances with antimutagenic activity (NOVICK and SZILARD 1952; WEBB and KUBITSCHEK 1963; MAGNI, VON BORSTEL and SORA 1964) are limited to two classes of compounds, purine ribosides and acridines, neither of which were observed previously to have a specific anti-s.p. mutagenic activity. (We except CLARKE's [1964] example of antimutagenesis since it seems a special case). Because the acridines are, with the methyl purines, potent inhibitors of dark repair one might suspect that they too may inhibit s.p. mutation, perhaps by substrate inhibition.

The accumulation of mutants in the absence of cell division has been reported in cells of a number of other organisms including stored seeds, Drosophila sperm and Serratia (STUBBE 1935, 1936; BLAKESLEE 1954) suggesting that stationary phase mutation is not limited to *E. coli*. One imagines that quantitatively s.p. mutation might be of more importance than replication mutation as a source of genetic variance in the long lived and slowly dividing cells of higher organisms than in bacteria. For this reason it could be interesting to see if s.p. mutation in other species is also suppressed by inhibitors of dark repair such as caffeine and the other methyl xanthines.

SUMMARY

In stationary phase (s.p.) cultures of *E. coli* 15 the *his*$^- \rightarrow$ *his*$^+$ mutation is reduced more than 90% in the presence of .008 M caffeine and to a lesser extent by .004 M caffeine; the same medium has no effect on the rate of mutation during normal growth. Inhibition of s.p. mutation is relieved by diluting the caffeine in the cultures sixfold with fresh medium. At the concentrations used caffeine does not alter the viable count or the glucose uptake of the cultures, and control tests make it unlikely that its effect was due to interference with the normal growth of prototrophs. It is suggested that s.p. mutation is dependent on the same enzymatic nucleotide replacement system that is operative in the dark repair process of ultraviolet damage, which is known to be inhibited by caffeine.

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