INDUCTION OF MUTATION IN CHLORAMPHENICOL-INHIBITED BACTERIA[1]

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In many bacteria chloramphenicol suppresses the synthesis of protein while having no immediate effect on the rate of synthesis of nucleic acid (Gale and Folkes, 1953; Wisseman *et al.*, 1954). This separation of the formation of nucleic acid and of protein permits a variety of experiments on the respective roles of nucleic acid and protein synthesis in fundamental biological phenomena. In the present work, the question of whether mutations can occur in bacteria during chloramphenicol inhibition has been studied. The production of mutations under these conditions would indicate that the synthesis of nucleic acid provides sufficient condition for the occurrence of mutations and that the nucleic acid formed during chloramphenicol inhibition is genetically functional.

That apparently normal deoxyribonucleic acid (DNA) can be formed during chloramphenicol inhibition has already been shown in the case of bacteria infected with phage (Hershey and Melechen, 1957; Hershey *et al.*, 1958; Tomizawa and Sunakawa, 1956; Tomizawa, 1958). The DNA formed under these conditions is found, like normal DNA, in the mature phage formed after removal of the chloramphenicol.

It was hoped that the present investigation would show whether in bacteria also biologically functional nucleic acid can be formed in the absence of protein synthesis. Investigations using chemical and physical methods for the examination of the bacterial nucleic acids formed in the presence of chloramphenicol indicate that the RNA (ribonucleic acid) differs in several respects from normal RNA. Under certain conditions some of the RNA formed in the presence of chloramphenicol is broken down after chloramphenicol is removed (Hahn *et al.*, 1957; Neidhardt and Gros, 1957; Horiuchi *et al.*, 1958). Also part of the RNA formed in inhibited bacteria is distinguishable electrophoretically from normal RNA (Pardee *et al.*, 1957). There is some difference of opinion about the stability of the DNA formed in chloramphenicol-inhibited bacteria. Some authors (Neidhardt and Gros, 1957; Horiuchi *et al.*, 1958) report that the DNA is not subsequently broken down, whereas others (Hahn *et al.*, 1957) claim that the DNA, like RNA, is unstable.

In any event, the identification of the nucleic acid formed during chloramphenicol inhibition as normal nucleic acid requires a biological test. If mutations occur in bacteria during chloramphenicol inhibition, the argument can be made not only that nucleic acid synthesis is sufficient to permit mutation but also that the nucleic acid made under these conditions is genetically functional. Current opinion is that mutations would be expected to occur in the DNA rather than the RNA, and it will be presumed here that the genetically functional nucleic acid is DNA.

In the experiments to be described *Escherichia coli* was used, and the mutation observed was to resistance to the bacteriophage T5. Since the spontaneous mutation rate is low, it would be difficult to detect the spontaneous mutations occurring during the relatively short time that nucleic acid continues to be formed after addition of chloramphenicol. These would represent a very small additional number of mutants as compared to the relatively large number of mutants present initially. To increase the expected effect, caffeine was added to the bacterial culture with the chloramphenicol, earlier work of Novick and Szilard (1951) having shown that, with *E. coli* strain B growing in the chemostat, caffeine at a concentration of 150 μg per ml increases the rate of mutation to resistance to phage T5 more than 10-fold.

MATERIALS AND METHODS

The variant B/r of *E. coli* strain B (Witkin, 1947) was used in all experiments. The bacteria were grown at 37 C in flasks shaken to provide aeration. The synthetic medium used (Friedlein)

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contained, in g per L: NH_4Cl, 1; Na_2HPO_4, 3.5; KH_2PO_4, 1.5; $MgSO_4$, 0.1; lactic acid, 10.6; and NaOH, 3.5.

The optical density (OD) of bacterial suspensions was measured in 1 cm cuvettes in a Beckman DU spectrophotometer at 350 mμ. A calibration curve relating OD and viable count was established and later used to determine the bacterial density from the OD observed in a given experiment. An OD of 0.1 corresponded to 1.25×10^8 bacteria per ml. Since the relationship was linear for OD less than 0.3, samples were diluted to below 0.3 for measurement when necessary.

Mutants resistant to the virus T5 were scored by mixing bacteria with excess virus in 2.5 ml soft agar (0.8 per cent) and pouring this mixture as a thin layer over about 20 ml of hardened nutrient broth agar in petri dishes. Iron (ferrous ammonium sulfate) was added to the agar to a concentration of 5 μg per ml.[2] Mutant colonies were counted after 24 hr incubation at 37 C.

For chemical determinations of DNA, 10-ml samples were centrifuged in the cold, and the pellet was assayed for DNA with the diphenylamine reagent (Racker, 1952). For protein measurements, 1-ml samples were similarly prepared and assayed with the Folin-Ciocalteau reagent (Lowry *et al.*, 1951). Increase in protein was also measured by the uptake of C^{14}-labeled phenylalanine. This was done by adding 0.2 ml of a dilute solution of phenylalanine containing 0.5 μc per ml to 20 ml of bacterial culture. At intervals, 2-ml samples were filtered on Millipore (Millipore Filter Corporation, Watertown, New York) filters and washed three times with 5 ml of medium containing chloramphenicol and unlabeled phenylalanine. In one case the filters were washed with hot trichloracetic acid. The filters were dried and counted in an internal flow counter.

The experimental procedure, which is outlined in figure 1, was as follows. From a number of independent bacterial cultures started from small inocula, the one having the lowest frequency of

[2] With insufficient iron, T5 resistant mutants grown in the presence of T5 show two types of colony, normal sized and very small. When iron is added, all of the mutants give normal sized colonies. The ratio of the two types of colonies remains constant whether the mutations occur spontaneously or are induced by chemicals or radiations (Philip Marcus and Aaron Novick, *unpublished observations*).

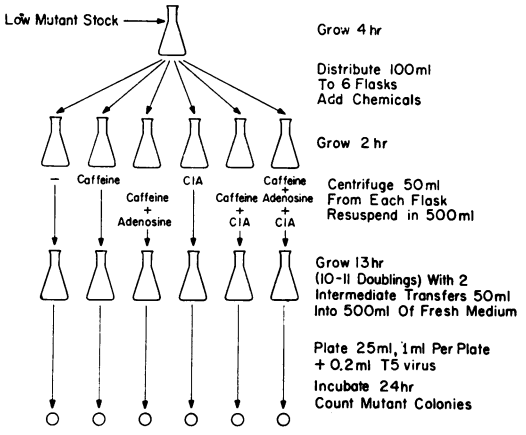

Figure 1. Diagram of experimental procedure. (Chloramphenicol abbreviated as ClA.)

T5 resistant mutants was selected and used to prepare a larger, actively growing culture. This latter culture was divided among a number of flasks to form a series of identical cultures. To these, appropriate additions were made, for example, to flask 1, nothing; to flask 2, caffeine; to flask 3, chloramphenicol; to flask 4, caffeine and chloramphenicol. The chemicals were allowed to act for 2 hr and then were removed by centrifugation of the bacteria. Following resuspension and growth in fresh medium (with two intermediate transfers to avoid high densities) to allow phenotypic expression of any induced mutations, samples were plated to determine the mutant density. The difference in mutant density between flasks 2 and 1 would show the mutagenic effect of caffeine on normally growing bacteria whereas the difference between flasks 4 and 3 would show the effect of caffeine on chloramphenicol-inhibited bacteria.

The precision of this procedure is limited by pipetting errors and sampling errors introduced each time a transfer is made. Sampling errors were reduced by always using large inocula in subcultures. The only significant pipetting error is that associated with plating the samples to determine the frequency of mutants. To avoid the errors introduced by pipetting many 1-ml samples, 25 ml were withdrawn with a volumetric pipette to a sterile flask from which 1-ml amounts were distributed to tubes containing soft agar and T5 virus. The flask was then rinsed several times with fresh medium and the rinsings also plated with T5. Division of the total colony count of all of the plates by 25 gave the number

of mutants per ml. With this method the pipetting error should be less than 1 per cent. The sampling errors can be estimated from the sample size transferred or counted. The expected uncertainty from both these causes is indicated in the tables.

Another possible error would be that produced if there were a significant difference in growth rate between mutant bacteria and wild type or if any of the treatments employed affected differently the viability of mutant bacteria. To examine the magnitude of such effects, artificial mixtures of mutant and wild type were subjected to the various experimental procedures. The frequency of mutants in such mixtures was made so large that any change in mutant frequency would have to be ascribed to a differential effect on viability or growth rate. Measurements made before and after the mixtures were carried through the whole procedure indicated that any difference was less than 3 per cent.

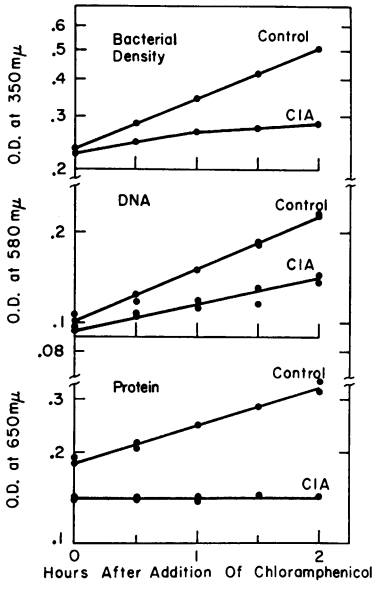

Figure 2. Optical density (OD) and chemical determinations of deoxyribonucleic acid (DNA) and protein in control and chloramphenicol-inhibited (ClA) cultures.

Effect of chloramphenicol. Two concentrations of chloramphenicol were used, 8 μg per ml and 20 μg per ml. Under these conditions, nucleic acid synthesis continued for about 2 hr following the addition of chloramphenicol, with little formed thereafter. There is, during these 2 hr, an increase in DNA of about 25 per cent (range 20 to 40 per cent) of the normal increase for the same period, where the normal doubling time is about 1.5 hr. During these 2 hr there is no apparent increase in protein. Typical results of the chemical determinations of DNA and protein are shown in figure 2.

There was no observable increase in protein, but measurement of the incorporation of radioactive phenylalanine into material insoluble in hot trichloracetic acid showed an incorporation in chloramphenicol-inhibited cultures of up to 7 per cent of that found in control cultures. It is not known whether this incorporation has its origin in some kind of exchange or turnover, or whether it results from the synthesis, in the presence of chloramphenicol, of a small amount of protein rich in phenylalanine.

To make sure that chloramphenicol is inhibitory but not lethal at these concentrations, viable

TABLE 1

Detailed data for two typical experiments. OD is at time of plating.

Expt IV (8 μg/ml Chloramphenicol)	Flask 1 (Control)	Flask 2 (Caffeine)	Flask 3 (Caffeine and Adenosine)	Flask 4 (Chloramphenicol)	Flask 5 (Caffeine and Chloramphenicol)	Flask 6 (Caffeine Adenosine and Chloramphenicol)
OD at 350 mμ	.236	.237	.233	.246	.229	.247
Colonies counted (25 ml)	4436	6489	4464	5083	5582	4999
Mutants/10^8	59.9 ± 1.4	87.4 ± 1.9	60.9 ± 1.4	65.8 ± 1.5	77.8 ± 2.0	64.5 ± 1.5
Expt V (20 μg/ml Chloramphenicol)						
OD at 350 mμ	.968	.992		.884	.852	
Colonies counted (10 ml)	7132	11159		7041	7952	
Mutants/10^8	58.6 ± 1.4	89.7 ± 1.9		63.6 ± 1.5	74.2 ± 2.0	

TABLE 2

Summary of results

Expt	Normal (2-1)	Chloramphenicol (5-4)	Normal and Adenosine (3-1)	Chloramphenicol and Adenosine (6-4)	Caffeine added 8 min after Chloramphenicol
I	28.8 ± 2.3	10.0 ± 2.6			
II	30.1 ± 2.3	13.3 ± 2.6			
III	29.0 ± 2.3	9.7 ± 2.6	−3.5 ± 2.3	−8.1 ± 2.6	
IV	27.5 ± 2.3	12.0 ± 2.6	+1.0 ± 2.3	−1.3 ± 2.6	
V	31.1 ± 2.3	10.6 ± 2.6			12.3 ± 2.6

Summary of results showing the effects of caffeine and adenosine on the induction of mutation in normal and in chloramphenicol-inhibited cultures. Each number is a difference in frequency of mutants between two flasks, identical except that caffeine was present during the period of treatment in one and not in the other. These differences as indicated in the parentheses are taken from the appropriate columns shown in table 1. Thus the column labeled normal gives the number of mutants per 10^8 bacteria induced by caffeine in a normal culture and the column labeled chloramphenicol gives the number induced by caffeine in a chloramphenicol-inhibited culture. The next two columns show the effect of adenosine on the induction of these mutations. The last column gives the results of one experiment where caffeine was added 8 min after chloramphenicol.

counts were made at the time of addition of chloramphenicol and again after 2 hr. Rather than a decrease in viable count, an increase of about 10 per cent was found, perhaps resulting from the delayed separation of bacteria attached at the time of addition of the drug.

Effect of caffeine and chloramphenicol. Five experiments were performed following the protocol given in figure 1. Table 1 shows the results of two of these in detail, while table 2 summarizes all five experiments.

It may be seen from these tables that exposure to caffeine (at a concentration of 200 μg per ml) for 2 hr increases the number of mutants in normal cultures by about 30 per 10^8 bacteria, whereas in chloramphenicol-inhibited cultures caffeine gives an increase of about 10 per 10^8 bacteria. If these observations are compared with the chemical determinations of DNA in normal and in inhibited cultures, it appears that the number of mutations produced is about the same per unit of DNA formed in both cases. Thus the rate of induction of mutations per unit of DNA formed is comparable in normal and chloramphenicol-inhibited cultures.

It is known that certain ribosides such as adenosine counteract very strongly the mutagenic action of caffeine and even reduce the spontaneous mutation rate (Novick and Szilard, 1952). To see if the antimutagenic effect of adenosine could also be expressed without protein synthesis, in two of these experiments, two additional flasks were used, one containing caffeine and adenosine, the other chloramphenicol, caffeine, and adenosine. Table 2 shows that adenosine (at a concentration of 100 μg per ml) completely suppresses the effect of caffeine in both cases.

When the caffeine and chloramphenicol were added at the same time, there was a possibility that the effect of chloramphenicol was not immediate, and that some protein was formed in the presence of caffeine. Therefore in one experiment chloramphenicol was allowed to act for 8 min before the addition of caffeine. As may be seen in table 2, there was no suppression of the induction of mutations in this culture.

TABLE 3

An attempt to induce mutation in nongrowing cultures

Growth Conditions	Control	Caffeine
Chilled 2 hr		
OD at 350 mμ at time of plating	.290	.290
Number of mutant colonies counted	7747	8045
Mutants/10^8	84.9	88.2
Incubated in buffer for 2 hr		
OD at 350 mμ	.294	.278
Colonies counted	7882	7439
Mutants/10^8	85.2	85.0
Normal growth for 2 hr		
OD at 350 mμ	.284	.288
Colonies counted	7810	9855
Mutants/10^8	87.5	108.6

To determine whether nucleic acid synthesis is a necessary as well as a sufficient condition for the occurrence of caffeine-induced mutations, the basic experiment was modified to suppress all synthesis. This was done by incubating the bacteria in buffer instead of the usual Friedlein's medium plus chloramphenicol, or by keeping the bacteria at 4 C in Friedlein's medium during the treatment period. There is no induction of mutations under these conditions (table 3), whereas mutations are induced in a culture growing normally during the same time.

DISCUSSION

These experiments show that mutations can be induced in bacterial cultures inhibited by chloramphenicol and that mutations are induced at about the same rate per unit of DNA formed as in uninhibited cultures. This suggests that it is DNA synthesis alone, rather than protein synthesis alone or some combination of protein and DNA synthesis, that is necessary for the occurrence of mutations. Furthermore, the DNA formed during chloramphenicol inhibition must be good nucleic acid in the sense that it is genetically functional, i. e., it perpetuates mutations occurring in it. These results are similar to the recent finding by Brenner and Smith (1957, *personal communication*) that 5-bromuracil present during the chloramphenicol inhibition of bacteria infected with phage T2 produces a large number of r mutants in the phage released after removal of chloramphenicol.

The present conclusions are subject to some qualification. In the first place, although there is very little, if any, net increase in protein, phenylalanine is incorporated into protein at a rate which cannot be disregarded. As a result the possibility that mutations occur during some kind of protein turnover cannot be excluded, nor can the possibility that mutations require the formation of a protein which is made in the presence of chloramphenicol and is only an inappreciable fraction of the total protein. In the second place, the assumption has been made that it is the formation of DNA rather than of RNA which leads to the occurrence of mutations. It might be possible to verify this assumption by experiments under conditions where only RNA is made, as in the case of W-6, a methionine-requiring strain of *E. coli*, which continues to make RNA, but not DNA, when deprived of methionine (Borek and Ryan, 1958).

There are other possible explanations of the observed facts which must be considered. For example, caffeine might be concentrated in the bacterial cell and produce mutations in the bacteria when they are transferred to medium lacking chloramphenicol and caffeine. However, the work of Koch (1956) with radioactive caffeine indicates that caffeine is not fixed in any detectable way by *E. coli*.

In many cases, mutagenic agents apparently do not directly induce mutations. For example, there is evidence that ultraviolet light produces chemical alterations which lead to the production of mutations when the organisms are subsequently allowed to reproduce (Novick and Szilard, 1949). It is possible that the mutations induced by caffeine do not occur during the treatment period and result from some modification of the genetic structure during treatment which makes subsequent mutation probable. It must be remarked, however, that the mutagenic action of caffeine requires nucleic acid synthesis while caffeine is present, whereas ultraviolet light and other similar reactive agents can produce their effect under conditions in which the bacteria are completely dormant. Thus, it is likely that the effect of caffeine is directly related to the mutational event.

It might be thought that the effects of caffeine and adenosine are due to some induced change, resulting from the presence of these compounds, in the level of an enzyme or enzymes related to nucleic acid formation. Their structural resemblance to end products of these enzymes might cause such an effect, as has been observed with several pyrimidines (Yates and Pardee, 1957). Since there is little, if any, protein synthesized in the presence of chloramphenicol, it is unlikely that the effects of caffeine and adenosine occur by way of a change in an enzyme level.

There are several ways caffeine and adenosine could produce their effects on the DNA formed during chloramphenicol treatment. If it is assumed that a mutation results from the substitution of one purine or pyrimidine base for another, it might be imagined that caffeine makes more likely the insertion of the wrong base and adenosine of the correct base. Alternatively, the effects might be less direct. For example, it has been suggested (Koch and Lamont, 1956) that the

effect of caffeine is on the activity of some enzyme involved in nucleic acid synthesis, the resulting lack of balance making more likely an error in a base insertion. How adenosine would reverse such an effect is not clear.

The present results provide some insight into the nature of a bacterial chromosome, should it exist. In bacteriophage, it is known from the work of Hershey and Chase (1952) and Hershey (1957) among others that the genetic material is essentially pure DNA. If phage can be said to have a chromosome, it must be composed of DNA only. Likewise the present experiments suggest that the formation of the essential macromolecular organization of DNA in bacteria is independent of any protein. If a chromosome does exist in bacteria, either it contains no protein, or if it does, then the protein can be added after the specific DNA has been independently formed.

SUMMARY

Mutations can be induced by caffeine in chloramphenicol-inhibited bacteria (*Escherichia coli*), where nucleic acid, but not protein, is being synthesized. These mutations occur at about the same frequency per unit of deoxyribonucleic acid formed as in control uninhibited cultures. As in control cultures the mutagenicity of caffeine can be suppressed by the addition of adenosine.

It is concluded from these results that the deoxyribonucleic acid formed in chloramphenicol-inhibited bacteria is genetically functional and that the synthesis of this deoxyribonucleic acid is sufficient condition for the induction of mutations.

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