CHARACTERIZATION OF TWO BACTERIAL MUTANTS WITH TEMPERATURE-SENSITIVE SYNTHESIS OF DNA

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DESPITE considerable interest in recent years, the replication of bacterial DNA and the mechanism of its regulation are still poorly understood, as can be seen in recent reviews of the subject (LARK 1966; MAALØE and KJELDGAARD 1966). One approach to the study of essential cellular syntheses is through the study of temperature-sensitive mutants (APIRION 1966; EPSTEIN *et al.*, 1963; NEIDHARDT 1966). Already several papers have appeared describing bacterial mutants impaired in synthesis of DNA at higher temperature (BONHOEFFER 1966; KOHIYAMA *et al.* 1966; MENDELSON and GROSS 1967). The present report describes a procedure for the isolation of bacterial mutants with temperature-sensitive synthesis of DNA and the characterization of two mutants representative of the types which were most frequently encountered. The mutants studied resemble the two classes found previously (KOHIYAMA *et al.* 1966); but from the experiments presented here, it is evident that at least one of them represents a new kind of block in DNA synthesis. A preliminary report of these mutants has been presented (FANGMAN 1966).

MATERIALS AND METHODS

Bacteria: The wild-type strain of *Escherichia coli* used for isolation of temperature-sensitive mutants was K12SH-28, a thymidine phosphorylase negative, F^- prototroph previously described. Incorporation of radioisotopically-labeled thymidine is a quantitative measure of DNA synthesis in this strain (FANGMAN and NOVICK 1966). Strain AB2297 is an Hfr obtained originally from DR. E. A. ADELBERG. It injects its chromosome during mating in the order *origin . . . str . . . mal . . . xyl . . . ilv*. AB2297 requires isoleucine, valine, thiamine and adenine, and is unable to utilize xylose.

Media: Glucose-minimal medium is the F-buffer of SADLER and NOVICK (1965) supplemented with 4 mg/ml glucose. Casamino acids-glucose-minimal medium contains, in addition, 2 mg/ml Difco vitamin-free casamino acids. Other supplements are amino acids (40 μg/ml each), adenosine (20 μg/ml) and thiamine (5 μg/ml). LB broth contains, per liter, 12 g Difco tryptone, 5 g Difco yeast extract, 10 g NaCl and 1 g glucose. BH broth is the brain-heart infusion medium described by EIDLIC and NEIDHARDT (1965) but contains, in addition, 10 g/l glycerol. Agar plates were prepared by adding 15 g/l Difco agar.

Culture methods: Temperature shifts were accomplished by diluting cultures 1:4 into media or buffer prewarmed to the new temperature. Experiments in which cells were incubated in buffer employed unsupplemented minimal medium (F-buffer) as the buffer. Culture growth was followed spectrophotometrically at 350 mμ (supplemented or unsupplemented minimal medium) or at 600 mμ (broth media). Viable counts were determined on BH broth-agar by the spread plate technique.

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Mutant isolation: Growing cells of strain K12SH-28 incorporate thymidine fairly efficiently into their DNA (FANGMAN and NOVICK 1966). When the compound is provided as H^3-thymidine of high specific radioactivity these cells, when subsequently stored at low temperature, lose viability as a result of the tritium decay. Mutant bacteria unable to synthesize DNA under the conditions imposed during the presence of H^3-thymidine in the medium should not be affected in this way. This formed the basis for enrichment of mutants defective in DNA synthesis at 37°C.

A culture of bacteria growing in casamino acids-glucose-minimal medium was incubated for two hours at 25°C with the mutagen ethyl methanesulfonate (1%). After intermediate cultivation at 25°C in the same medium in the absence of mutagen, an exponential phase culture was shifted to 37°C and after 2 to 60 min, thymidine-methyl-H^3 (13.7 mc/μmole, New England Nuclear) was added to 1 μg/ml. Incubation was continued for one to two hrs at 37°C and the culture chilled. The cells were washed several times with buffer and resuspended at a titre of about 10^7 cells/ml in buffer containing 10% glycerol. The suspension was distributed into a series of tubes and frozen at —20°C. Periodically, samples were thawed and plated for viable counts. When the viability had declined by a factor of 10^3 to 10^4 (several weeks) the suspension was diluted and plated on BH broth agar and incubated at 25°C. Colonies which appeared on an agar plate were replica plated onto two BH broth-agar plates; one was incubated at 25°C and one at 37°C. In a small number (six) of such mutant searches the following was observed: Approximately one temperature-sensitive clone was found per 10^3 clones replica plated. Of the temperature-sensitive isolates from a given search that were examined, approximately 5–10% were mutants of the type described in this paper.

Auxotrophic mutants were isolated by penicillin enrichment after ethyl methanesulfonate mutagenesis.

C^{14}-thymidine incorporation: For experiments in which DNA synthesis was measured by C^{14}-thymidine labeling, 5 μg/ml thymidine-3-C^{14} (.05 μc/μg; New England Nuclear) was added to the medium. Samples were taken into an equal volume of cold 10% trichloroacetic acid containing 2 mg/ml C^{12}-thymidine. After standing on ice at least 30 minutes the samples were filtered through membrane filters (Gelman GA-6, 24 mm) and the precipitates washed on the filters with two 10 ml volumes of cold water. Radioactivity was determined in a liquid scintillation counter.

Chemical determinations: RNA was determined by the orcinol method (SCHNEIDER 1945), protein by the phenol method (LOWRY *et al.* 1951), and DNA by a modified diphenylamine method (GILES and MYERS 1965). These determinations were carried out on cold 5% trichloroacetic acid washed cell samples.

Determination of nucleoside triphosphates: For determination of nucleotide pools, cells were grown several generations at 25°C in the low phosphate Tris-glucose medium of EDLIN and MAALØE (1965) containing P^{32}-orthophosphate (100 μc/μmole). The cultures were then diluted into the same medium at 39°C and samples taken at intervals. Culture samples were rapidly filtered on membrane filters (Gelman GA-6, 24 mm) and the filter wetted with 0.5 ml cold 0.5 N perchloric acid. After extraction in the cold for 30 min the precipitated cells were removed by centrifugation and the perchloric acid removed by addition of KOH. Separation of nucleoside triphosphates in the samples was performed on poly(ethyleneimine) cellulose-coated plastic sheets prepared as described by RANDERATH and RANDERATH (1966). Chromatography was two dimensional (procedure 2 of NEUHARD and MUNCH-PETERSON 1966), resulting in separation of the eight nucleoside triphosphates. For each sample chromatographed, coincidence of radioactivity and carrier nucleotides was verified by autoradiography. Spots corresponding to the nucleotides were cut out of the chromatogram and radioactivity determined directly in a liquid scintillation counter.

Enzyme assays: β-galactosidase was determined in toluene-treated culture samples as previously described (HORIUCHI, TOMIZAWA and NOVICK 1962). Cell-free extracts were prepared from bacteria washed and resuspended with .05 M Tris-HCl, pH 7.5–.01 M 2-mercaptoethanol by rupture in a French pressure cell, followed by centrifugation to remove cell debris. DNA polymerase activity was assayed in cell-free extracts with the system described by RICHARDSON *et al.* (1964) employing activated calf thymus DNA, except that the buffer was Tris-HCl, pH 7.5.

The labeled substrate was α-P^{32}-dATP (International Chemical and Nuclear Corp.). Deoxyribonucleoside monophosphate kinase activities were measured by substituting individual unlabeled deoxyribonucleoside monophosphates or P^{32}-TMP in the DNA polymerase assay where polymerase activity is in excess.

Bacteriophage growth: The media and techniques used for preparation and assay of T4B are in the literature (SÉCHAUD *et al.* 1965). Tryptone broth was the medium employed for the experiment reported in Table 4. Lysates of λvir 1210, a virulent strain of λ, were prepared and assayed on tryptone broth-agar plates (SÉCHAUD *et al.* 1965). Growth medium for the bacteria used in λvir 1210 experiments was M9 (SÉCHAUD *et al.* 1965) supplemented with, per liter, 15 g decolorized casamino acids, 0.5 g NaCl, 0.6 g $MgSO_4$ and 2 g maltose. For the experiment reported in Table 5, phage in TM buffer (.01 M $MgSO_4$–0.01 M Tris-HCl, pH 7.4) were added to bacteria (5×10^8/ml) prewarmed to 39°C in TM buffer. After 15 minutes the mixture was diluted 10 fold into 39°C growth medium containing C^{14}-thymidine.

Bacterial matings: Mating experiments were carried out in LB broth by the methods described by TAYLOR and THOMAN (1964). Incubation was for 15 minutes at 37°C followed by 2.5 hrs at 25°C. The bacteria were then washed with buffer and plated on appropriately supplemented glucose-minimal agar and incubated at 39°C.

RESULTS

Synthesis of macromolecules at higher temperature: Mutants FA21 and FA22 show a behavior after shift to higher temperature which is typical of two kinds of DNA-defective mutants most frequently encountered in a series of searches. When cultures of these mutants growing at 25°C are transferred to 39°C culture optical density increases initially at near wild-type rates (Figure 1). However,

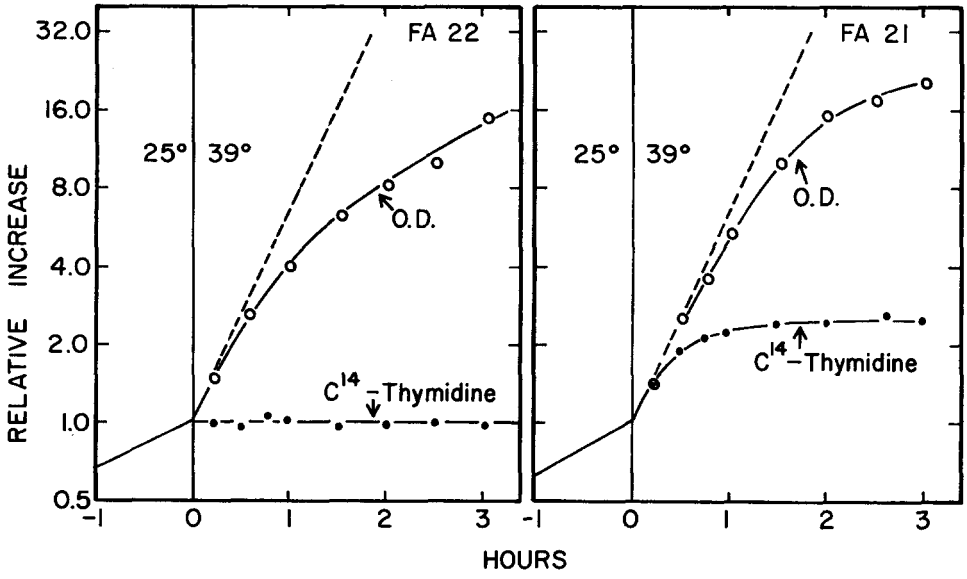

FIGURE 1.—C^{14}-thymidine incorporation and mass increase by cultures of mutants shifted from 25°C to 39°C. The medium was BH broth and the cultures were grown in the C^{14}-thymidine containing medium several generations at 25°C before the shift. The values are normalized to the values at zero time. The dashed lines represent data for both culture optical density and C^{14}-thymidine incorporation by the wild type.

TABLE 1

*Increase in mass, protein, RNA, DNA and total cells in cultures of mutants and wild type shifted to 39°C**

Strain	Time at 39°C (min)	Mass	Protein	RNA	DNA	Cells
K12SH–28 (wild)	0	1.0	1.0	1.0	1.0	1.0
	30	1.8	1.5	1.7	1.5	2.0
	60	3.6	2.8	3.6	3.6	3.7
	90	6.6	5.6	6.8	5.5	4.8
	120	13	9.9	13	10	9.8
FA22	0	1.0	1.0	1.0	1.0	1.0
	30	1.4	1.4	1.4	1.1	1.2
	60	2.4	2.2	2.3	1.2	1.1
	90	3.4	2.6	2.8	1.2	1.3
	120	4.2	3.2	3.2	1.3	1.3
FA21	0	1.0	1.0	1.0	1.0	1.0
	30	2.0	2.0	2.0	1.2	1.4
	60	3.2	4.0	3.9	1.5	1.9
	90	5.8	7.3	6.3	1.7	. .
	120	8.6	12	9.4	1.9	2.8

* Cultures growing in casamino acids-glucose-minimal medium at 25°C were shifted to 39°C in the same medium. Mass was measured as culture optical density at 350 mμ. Protein, RNA and DNA were measured by colorimetric assays (MATERIALS AND METHODS). Samples for total cells were taken into an equal volume of 10% formalin and counted under a phase contrast microscope with a Petroff-Hausser counter. All values are normalized to the value at zero time.

as shown in Figure 1, DNA synthesis in FA22 ceases immediately and in FA21 occurs at a near normal rate for approximately one hour and then stops. The data in Table 1 show that at 39°C there are increases in RNA and protein commensurate with the optical density increase. Moreover, formation of β-galactosidase can be induced with normal kinetics at higher temperature (data not shown) indicating that protein synthesis and messenger RNA metabolism are normal. These results show that DNA synthesis is very preferentially inhibited in the mutants.

When the kinetics of synthesis of DNA are compared for cultures shifted to a series of temperatures, a difference in behavior of the two mutants is still evident. In Figure 2B it can be seen that after transfer to higher temperatures strain FA21 shows an initial rate of DNA synthesis which is the same as the wild type, but synthesis stops earlier the higher the temperature. In contrast, strain FA22 upon transfer to higher temperature makes DNA at a fixed reduced rate whose value varies sharply with temperature (Figure 2A).

That no breakdown of DNA occurs after transfer of these mutants to higher temperature is suggested by the results in Table 1. Further experiments have shown that no appreciable turn-over of DNA occurs for at least three hours at 39°C (data not shown).

Effects on cell division and viability: In cultures of FA21 and FA22 at 39°C increase in cell number approximately parallels DNA increase (Table 1). The increase in culture optical density at 39°C, which can occur up to at least 20-fold,

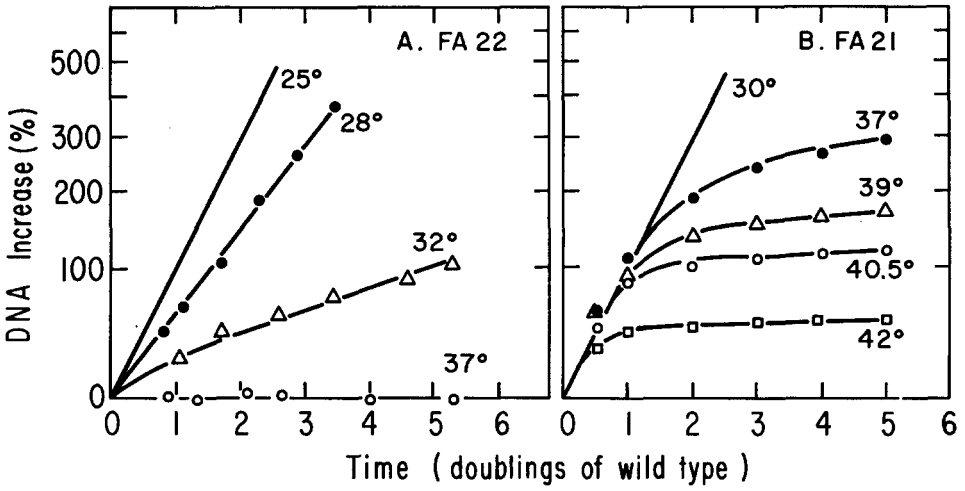

FIGURE 2.—Kinetics of DNA synthesis by cultures of mutants shifted from 25°C to various temperatures. DNA synthesis was measured by C^{14}-thymidine incorporation and the values are normalized to the values at zero time. The time scale is in doublings of the wild type. Data for the wild type by definition follow the 25°C or 30°C line for all temperatures. A. Data for mutant FA22. B. Data for mutant FA21.

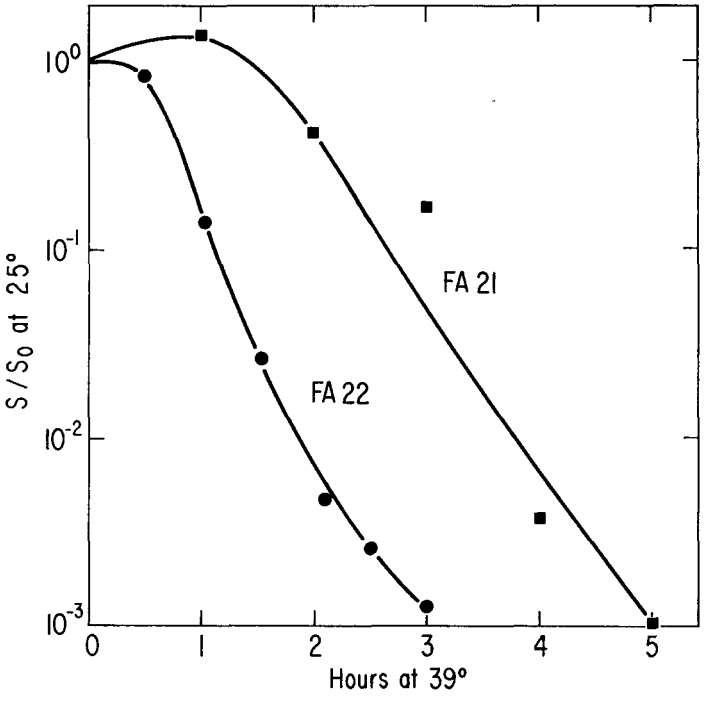

FIGURE 3.—Loss of colony forming ability at 25°C by cultures of mutants shifted to 39°C in casamino acids-glucose-minimal medium. The data are expressed as the total colony formers at any time (S) divided by the number of colony formers at zero time (S_0).

is reflected by an equivalent increase in cell length. In both strains there is, in addition, a rapid decline in viability associated with the arrest of DNA synthesis, as shown in Figure 3. One possible explanation would be that cells become fragile at 39°C and are killed during plating. This seems not to be the case, however, since a similar death rate is found when cells at 25°C are transferred to pre-warmed broth agar plates which are then cooled to 25°C at various intervals. In further experiments it was found that loss of viability is correlated with the increase in cell mass; conditions which slow formation of mass decrease the death rate. Starvation for a required amino acid in auxotrophic strains prevents death altogether.

These facts suggest that death is a consequence of a secondary event initiated by cessation of DNA synthesis. This interpretation is supported by the existence of mutants which can be isolated from FA22 which do not lose viability following arrest of DNA synthesis. The behavior of one of these, FA2206, is illustrated in Figure 4. FA2206 appears to retain all the other properties of FA22.

Recovery of DNA synthesis: For study of the recovery of DNA synthesis after high temperature treatment, a culture of FA22 growing at 25°C in BH broth was

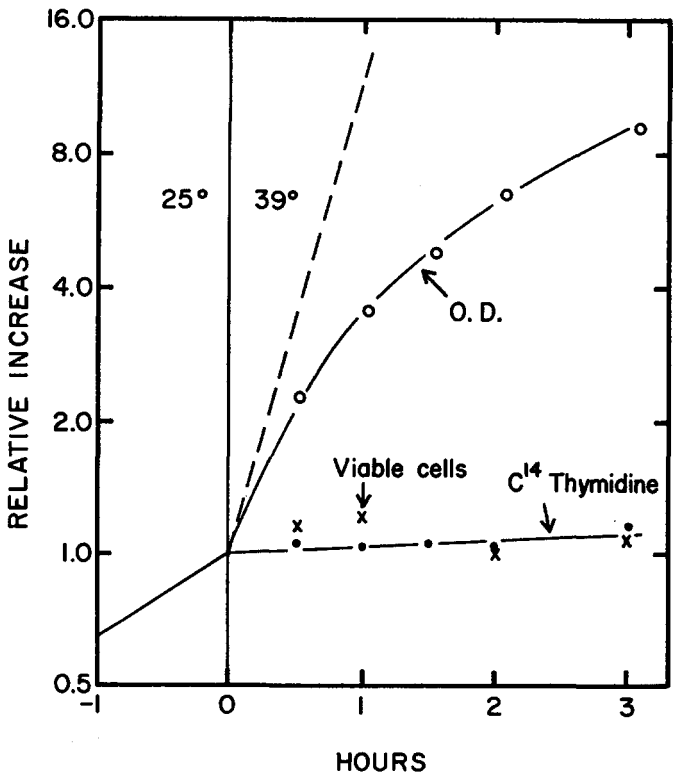

FIGURE 4.—C^{14}-thymidine incorporation and increase in mass and colony forming cells by a culture of mutant FA2206 shifted from 25°C to 39°C. Colony forming cells were determined by plating at 25°C (MATERIALS AND METHODS). Other details are in the legend of Figure 1.

transferred to 39°C for one hour and then returned to 25°C. Synthesis of DNA resumed very slowly, increasing at most by 30% after three hours. Recovery is more rapid in FA2206, the strain which remains viable, there being a doubling of DNA after three hours. The wild type, K12SH–28, when treated similarly, commences synthesis at the ultimate 25°C rate immediately, giving a four fold increase after three hours. In further experiments cultures of FA22 in BH broth at 25°C were washed, transferred to buffer at 39°C for one hour, and then returned to BH broth at 25°C. Synthesis of DNA resumed at the normal rate after a lag of about the same length (90–120 minutes) found with wild type treated similarly. To distinguish whether the resumption of synthesis in these experiments indicates a reversibility of the inactivation of the thermosensitive element or results from *de novo* synthesis of this element, presumably a protein, recovery of synthesis of DNA at 25°C was observed in medium containing chloramphenicol (100 μg/ml). From the results in Table 2, it can be seen that there is substantial recovery of synthesis of DNA in the presence of chloramphenicol, as if the effect of heating at 39°C on the thermolabile element is, at least in part, reversible.

Strain FA21 which continues to make DNA for a while after shift to 39°C shows a rapid recovery upon return to 25°C, and this recovery still occurs when protein synthesis is inhibited by chloramphenicol, as can be seen in Figure 5. Assuming that the temperature-sensitive element in FA21 is a protein, this experiment would indicate that inactivation of the element at 39°C is reversible. When the 39°C culture is returned to 25°C in media without chloramphenicol, synthesis of DNA resumes at a rate 50% greater than normally observed at 25°C, while culture optical density increases at the normal rate. The normal DNA:mass ratio is therefore quickly restored.

Biochemical basis: It appears that the lesions in FA21 and FA22 occur at some step in the synthesis of DNA after precursor supply since the deoxyribonucleoside triphosphates continue to be made after transfer to 39°C, as can be seen from the results reported in Table 3. Further it can be seen that there is little, if any, decrease in the level of these nucleotides; and, in fact, there is an increase in the levels of dATP and dTTP.

TABLE 2

*Net DNA synthesized by mutant FA22 in the presence of chloramphenicol at 25°C after incubation in buffer at 39°C**

Time in buffer at 39°C (min)	C^{14}-thymidine incorporated at 25°C (mμg/ml)
0	21
45	13
60	9.1
120	4.3

* The medium at 25°C was glucose-minimal; chloramphenicol (Parke, Davis Co.) was used at 100 μg/ml. Incorporation of C^{14}-thymidine was followed in each culture for several hours. The values presented are the plateau values.

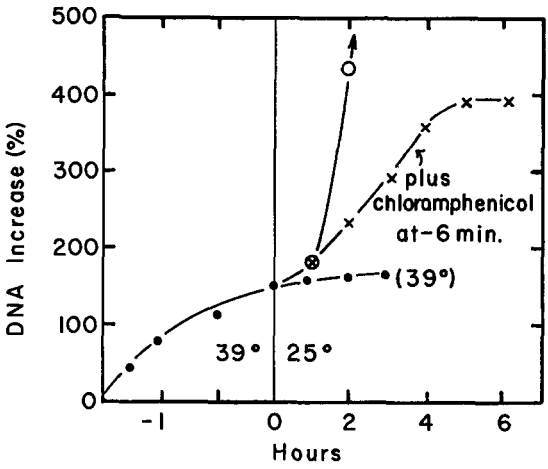

FIGURE 5.—Recovery of DNA synthesis at 25°C by a culture of FA21 previously transferred to 39°C. The data at 25°C were obtained with parallel cultures incubated at 39°C but which did not receive C^{14}-thymidine until transfer back to 25°C. All data were normalized to the amount of DNA present at the time of shift to 39°C. The rate of protein synthesis in the chloramphenicol treated culture, measured by C^{14}-arginine incorporation in another parallel culture, was less than 1% of that in the culture without chloramphenicol.

Several enzyme activities which are presumed to be necessary for cellular DNA replication were measured directly to see if they were thermolabile in the mutants. Crude cell-free extracts from cells grown at 25°C or after shift to 39°C for varying periods were assayed at 25°C and at 39°C, for the two mutant strains and for the wild type. No significant thermolability of DNA polymerase activity

TABLE 3

*Increase in mass and deoxyribonucleoside triphosphates in cultures of mutants and wild type shifted to 39°C**

Strain	Time at 39°C (min)	Mass	dATP	dCTP	dGTP	dTTP
K12SH–28 (wild)	0	1.0	1.0	1.0	1.0	1.0
	45	1.5	1.7	2.4	2.9	1.9
	90	2.5	3.4	5.4	3.4	2.6
	150	4.6	5.9	8.8	6.1	6.5
FA22	0	1.0	1.0	1.0	1.0	1.0
	45	1.6	5.1	3.4	2.5	4.1
	90	2.4	14.5	6.0	4.1	7.3
FA21	0	1.0	1.0	1.0	1.0	1.0
	45	1.6	2.0	2.4	1.5	1.8
	90	2.6	5.2	4.4	1.8	4.0
	150	4.4	7.6	8.3	4.0	11

* The technique for measurement of the deoxyribonucleoside triphosphates is given in MATERIALS AND METHODS; the values are normalized to the amount per ml of culture at zero time.

TABLE 4

*T4B stimulated DNA synthesis by wild type and mutants at 39°C**

Experiment	Strain	C^{14}-thymidine incorporated No phage	Plus T4B
		mμg/ml	
A	K12SH–28 (wild)	140	730
	FA22	3.3	364
B	K12SH–28 (wild)	166	1150
	FA21	2.9	1080

* Values presented are for incorporation after 30 min incubation with C^{14}-thymidine and phage. In experiment A the cultures were incubated 5 min at 39°C and in experiment B, 90 min at 39°C before addition of C^{14}-thymidine and phage. Incorporation values are normalized to initial culture densities of 0.10 at 600 mμ. Multiplicity of infection was about 3.0. Other details are in MATERIALS AND METHODS.

or of kinase activities for the four deoxyribonucleoside monophosphates was observed (see MATERIALS AND METHODS).

That the lesions do not preclude synthesis of all DNA molecules in the mutant cells is shown by the ability of phage T4B to make near normal quantities of DNA at 39°C, both in FA21 and FA22 (Table 4). On the other hand, the phage λ*vir* 1210 is unable to form DNA in these mutants at 39°C, as reported in Table 5, although adsorption of this phage is very good at 39°C. The wild-type bacterium supports λ*vir* growth, and therefore DNA synthesis, at 39°C (Table 5) and both FA21 and FA22 support the growth of this phage at 25°C. Evidently the thermolabile element in the mutant bacteria is needed for normal λ development.

Genetic mapping: Both strains FA21 and FA22 revert to wild type temperature-sensitivity at frequencies consistent with the existence of single mutations, cultures grown at 25°C having about 10^{-8} revertants when plated at 39°C. Although the exact genetic location of the mutations in FA21 and FA22 is unknown, the results of several bacterial matings indicate that both are linked to

TABLE 5

*DNA synthesized and phage yield by wild type and mutants infected with a virulent strain of λ at 39°C**

Strain	C^{14}-thymidine incorporated No phage	Plus λ*vir*	Phage yield
	mμg/ml		
K12SH–28 (wild)	335	110	54
FA22	<4	<4†	<1
FA21	<4	<4†	<1

* Values presented are for incorporation and phage yield after 80 min incubation in growth medium. K12SH–28 and FA 22 were grown at 25°C and FA21 was grown 90 min at 39°C before being washed and placed in TM buffer at 39°C for adsorption of phage (MATERIALS AND METHODS). Multiplicity of infection was about 4.0 and adsorption was greater than 90% in all three cultures. Phage yield is the number of phage per infected bacterium determined on chloroform lysed samples. The initial optical density of all three cultures was the same (0.30 at 600 mμ).

† Corresponds to less than 8 λ DNA equivalents per cell.

the locus for streptomycin resistance. For example, Hfr AB2297 was mated with streptomycin resistant derivatives of both mutants (see MATERIALS AND METHODS). and *ts*$^+$ *ade*$^+$ recombinants selected. Among 100 recombinants purified and tested, 90% from the FA22 mating and 78% from the FA21 mating received the streptomycin marker from the Hfr parent. Only about 50% of the recombinants from each mating received the xylose marker from the Hfr. Since 20 recombination units correspond to the length of chromosome transferred in approximately one minute (JACOB and WOLLMAN 1961; Low 1965), the mutations in FA22 and FA21 reside within about one minute of transfer from the locus for streptomycin resistance.

Physiological studies: In some respects strain FA21 resembles a mutant already reported (KOHIYAMA *et al.* 1966; KOHIYAMA *et al.* 1963) which makes a limited amount of DNA after transfer to higher temperature. Such behavior was interpreted on the basis of the presence of a thermosensitive initiator, a specific protein postulated to be necessary for a new round of replication of the bacterial genome (JACOB, BRENNER and CUZIN 1963; MAALØE 1963). The limited synthesis was compared to that which is observed in the wild-type strain after transfer to conditions where synthesis of protein is blocked, e.g., in the absence of a required amino acid or in the presence of chloramphenicol. Bacterial DNA molecules in the process of being replicated at the time the block is imposed are thought to finish replicating (LARK 1966; MAALØE 1963). The amount of DNA produced by strain FA21 at higher temperature is very temperature-dependent, and it is difficult to interpret this property as resulting from a thermosensitive initiator of DNA synthesis. Nevertheless, in an attempt to understand the nature of the limited synthesis in FA21 several experiments were performed and the results compared with those expected for a cell possessing a thermosensitive initiator.

Studies were first made of the limited synthesis of DNA observed after transfer of FA21 to higher temperature or upon addition of chloramphenicol to the wild type. As can be seen in Figure 6 the quantity of DNA made after transfer of a BH broth culture of FA21 to 39°C corresponds to that observed when the wild type is transferred to medium with chloramphenicol. But as can be seen from Figure 2B, the increment of DNA made by FA21 depends on the temperature used, and, therefore, the agreement at 39°C with chloramphenicol-inhibited wild type is apparently fortuitous. In addition, FA21 and chloramphenicol inhibited wild type differ in their response to media which afford different rates of growth. Media which give slower growth rates result in decreased increments of DNA being produced after transfer of FA21 to higher temperature or after addition of chloramphenicol to wild type (Table 6). The mutant usually synthesizes more DNA, however, and agreement is found only in media giving high growth rates. Furthermore, for the mutant the medium at 25°C has little effect on the amount of DNA made after transfer to different media at 39°C. When FA21 is transferred to nutritionally richer media at 39°C the rate of synthesis of DNA increases abruptly (data not shown), and the final increment is greater than for samples kept in minimal medium (Table 6). In contrast, the post-shift medium has no effect on the rate of synthesis of DNA or on the final increment of DNA

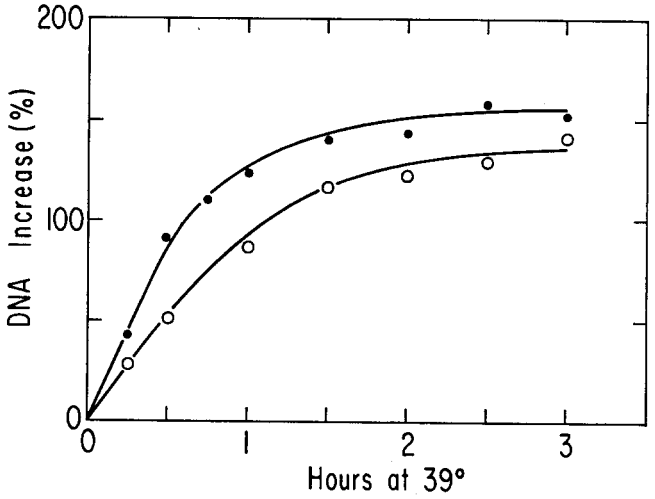

FIGURE 6.—DNA synthesis at 39°C by wild type inhibited with chloramphenicol and mutant FA21. The medium was BH broth and both strains had been grown several generations at 25°C in the medium containing C^{14}-thymidine. DNA increases in the chloramphenicol (50 μg/ml) treated culture of the wild type (○—○) and in the culture of the mutant (●—●) are shown.

in the case of the wild type inhibited by chloramphenicol. It should be noted, however, that the wild type when not inhibited by chloramphenicol exhibits a fairly abrupt increase in rate of synthesis of DNA after transfer to richer medium.

A possible explanation of the behavior of FA21 is that some enzyme involved in the synthesis of DNA loses activity slowly, complete inactivation requiring about 90 minutes at 39°C. This was tested by heating the mutant in buffer at 39°C to see if this would abolish the capacity to make DNA after return to

TABLE 6

DNA synthesized by mutant FA21 and chloramphenicol inhibited wild type at 39°C in different media

Medium		Normal 39°C doubling time of wild type (min)	Increment of DNA synthesized at 39°†	
			Mutant (percent)	Wild type plus chloramphenicol* (percent)
25°C	39°C			
BH broth	BH broth	22	200	140
casamino acids-glucose-minimal	casamino acids-glucose-minimal	30	178	72
glucose-minimal	glucose-minimal	60	165	57
glycerol-minimal	glycerol-minimal	75	104	39
glycerol-minimal	glucose-minimal	60	165	42
glycerol-minimal	casamino acids-glucose-minimal	30	198	40

* Chloramphenicol was used at 100 μg/ml.

† The DNA increment was determined by following C^{14}-thymidine incorporation until incorporation plateaus were reached in the cultures.

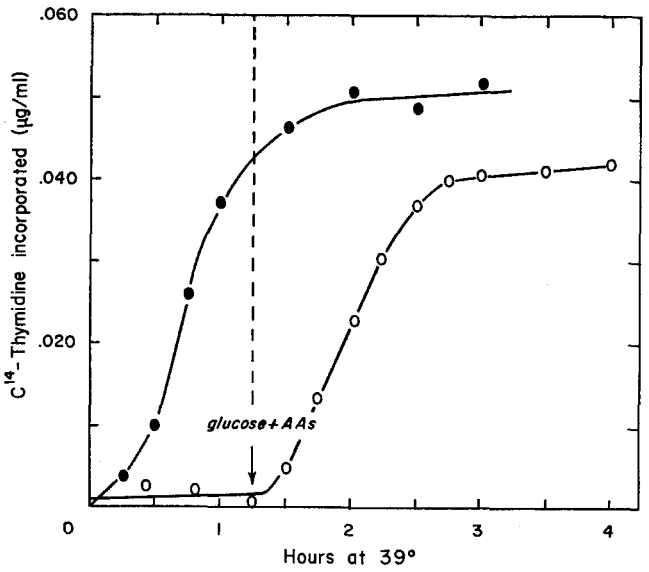

FIGURE 7.—DNA synthesis by mutant FA21 after incubation in buffer at 39°C. A casamino acids-glucose-minimal culture of FA21 growing at 25°C was washed and incubated in buffer at 25°C for 135 min; part was then shifted to 39°C in casamino acids-glucose-minimal medium (●—●) and part shifted to 39°C in buffer (O—O), both containing C^{14}-thymidine. After 75 min of incubation casamino acids and glucose were added to the buffer culture.

growth medium. To insure complete arrest of synthesis during heating, a washed culture of FA21 was first incubated in buffer at 25°C for 135 minutes by which time residual synthesis has stopped. The culture was then divided into two parts, both were shifted to 39°C, one into medium containing C^{14}-thymidine, and the other into buffer containing C^{14}-thymidine (Figure 7). After 75 minutes the culture in buffer was supplemented with glucose and casamino acid. By this time the control culture (medium) had almost attained its final increment of DNA. Yet in the supplemented buffer culture at this time, synthesis of DNA commenced at a normal rate and yielded almost the same final increment as the control culture (Figure 7). This shows that the limited synthesis of DNA in FA21 after transfer to higher temperature is not the result of a simple thermal inactivation of some agent involved in synthesis of DNA. The inactivation is apparent only when the cells are permitted to grow. The results are consistent with the behavior expected for a thermolabile initiator, i.e., despite inactivation of initiator during the heat treatment in buffer, rounds of replication of DNA already underway are completed when the cell is allowed to synthesize the necessary DNA precursors.

The result of an additional experiment also seemed consistent with that notion. After arrest of DNA synthesis at 39°C a culture of FA21 was cooled to 25°C for a short time (30 minutes) then returned to 39°C. The rationale of this experiment was that a short exposure to lower temperature would allow active temperature-sensitive element (presumably a protein) to be synthesized or renatured (see

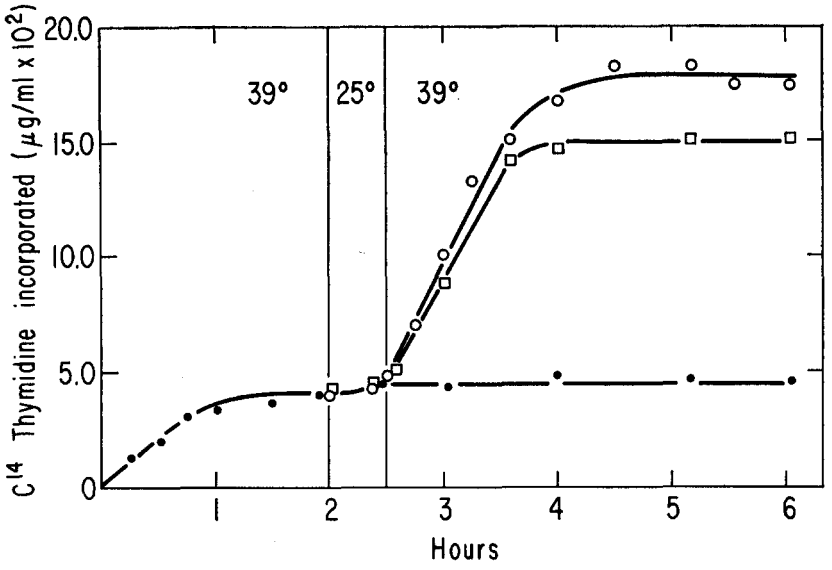

FIGURE 8.—DNA synthesis at 39°C after cooling a culture of FA21 in which synthesis had been arrested at 39°C. A casamino acids-glucose-minimal culture of FA21 growing at 25°C was transferred to 39°C medium containing C^{14}-thymidine. Incorporation by a culture always at 39°C (●—●), and portions cooled to 25°C for 30 min with (□—□) or without (○—○) chloramphenicol (100 μg/ml) is shown.

Figure 5), which, if it were involved with initiation of DNA synthesis, might be sufficient to start additional rounds of replication which would continue at 39°C. Figure 8 shows that DNA synthesis does resume at 39°C giving a rate of synthesis and increment of DNA which is two to three times that found during the initial 39°C treatment. The presence of chloramphenicol (100 μg/ml) at 25°C does not prevent the burst of DNA synthesis at 39°C (Figure 8) suggesting that the burst is not dependent on protein synthesis at 25°C. Moreover, neither starvation for uridine or leucine in a uridine-leucine auxotroph nor addition of 5-fluorodeoxyuridine (5 μg/ml) to inhibit DNA synthesis at 25°C has been found to markedly reduce the subsequent burst of DNA synthesis. Glucose starvation at 25°C does reduce it as much as 75% (data not shown), suggesting that the process is energy-dependent. The burst of synthesis at 39°C after cooling could represent initiation and completion of new DNA replication cycles.

If synthesis of DNA stops in FA21 because of inactivation of the presumed initiator then blocking protein synthesis at the lower temperature and allowing DNA synthesis to come to a halt should deplete the cell of the temperature-sensitive element. To test this a leucine auxotroph of FA21 (FA2105) was deprived of leucine at 25°C and formation of DNA observed (Figure 9). (FA2105 exhibits the typical pattern of DNA synthesis when starved for its required amino acid at 25°C or 39°C.) The 25°C leucine-starved culture was then transferred to 39°C, and one portion was provided with leucine. Protein synthesis, estimated from increase in optical density, resumed immediately. If the initiator

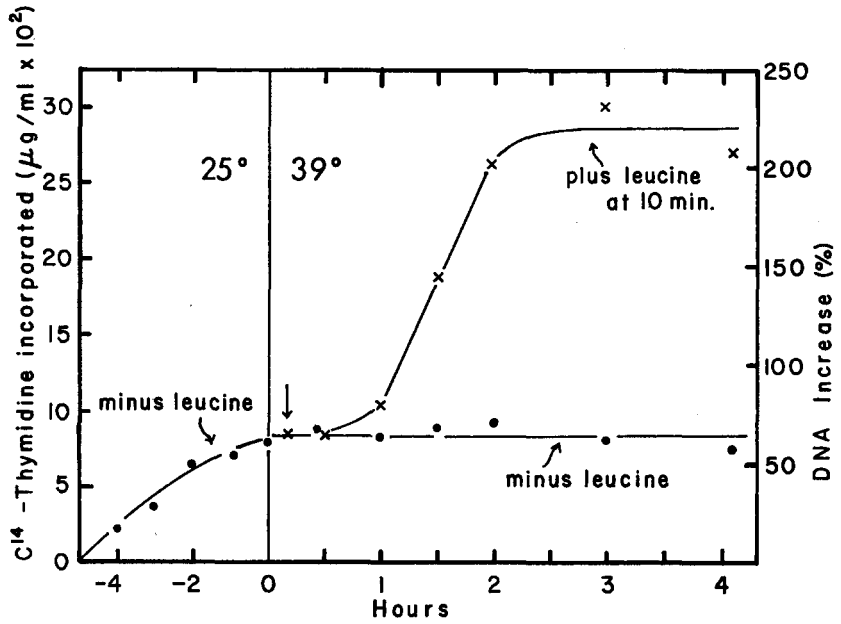

FIGURE 9.—DNA synthesis at 39°C by mutant FA21 after amino acid starvation at 25°C. The medium was glucose-minimal. The amino acid-starved culture at 25°C was shifted to the same medium at 39°C (●—●). Part of it received 40 μg/ml leucine after 10 min (×—×). C^{14}-thymidine was present since—5 hours at 25°C.

is depleted when synthesis of DNA ceases at 25°C and if it is rapidly inactivated in the mutant at 39°C, one would not expect synthesis of DNA to resume after transfer to 39°C plus leucine. But, as can be seen from Figure 9, after a short lag synthesis of DNA resumes at a high rate, and a large increment of DNA is produced. This resumption of DNA synthesis, as expected from the behavior of wild type treated similarly, is dependent on protein synthesis since addition of chloramphenicol (100 μg/ml) along with leucine prevents it. The thermolabile element in FA21 would appear not to correspond to the hypothetical initiator, though this experiment does not tell whether or not the element is involved with DNA initiation.

DISCUSSION

The characterization of temperature-sensitive mutants FA21 and FA22 indicates that as with other mutants (BONHOEFFER 1966; KOHIYAMA *et al.* 1966) the genetic lesions result in a preferential block of DNA synthesis since there is no evident immediate effect on RNA and protein synthesis. The blocks apparently occur at some biosynthetic step after precursor supply. Presumably, then, the mutations result in the alteration of elements directly involved with the DNA replication process. As in the case of other mutants (BONHOEFFER 1966; KOHIYAMA *et al.* 1966), the DNA polymerase appears unchanged in FA21 and FA22, and defects in other enzymatic or structural elements must be sought.

Since the rapid loss of viability which occurs when DNA synthesis is stopped in the mutants at higher temperature depends on RNA and protein synthesis, and since further mutations can occur which prevent the loss of viability, it is likely that death of the bacteria is the result of some secondary phenomenon induced by the arrest of synthesis of DNA. It is not surprising that excessive cell division does not occur in the absence of DNA synthesis in the mutants. Bacteria might be expected to have a control mechanism which couples these two synthetic processes so that cessation of DNA synthesis does not result in production of bacteria without genetic material.

The behavior of FA21 is not easily reconcilable with the idea that a protein needed only for the initiation of a round of DNA replication is thermolabile. In particular, the extent of synthesis of DNA after shift to higher temperature is very temperature-dependent (Figure 2) as though replication comes to a halt at some arbitrary point rather than at a fixed initiation point. Other experiments provide additional evidence which argues against the temperature-sensitive element in FA21 being the *initiator* of DNA replication which has been proposed by others (JACOB, BRENNER and CUZIN 1963; MAALØE 1963). First, amino acid starvation of the FA21 auxotroph which would be expected to deplete the bacteria of the presumed initiator does not deplete them of the temperature-sensitive agent (Figure 9). Second, after arrest of DNA synthesis at higher temperature, FA21 is unable to support the growth of phage λ (Table 5) which is thought to possess its own initiator (JACOB, BRENNER and CUZIN 1963). This is in contrast to a mutant interpreted as having a labile initiator in which λ can grow (KOHIYAMA *et al.* 1966). On the other hand the experiment in which cooling FA21 to 25°C for a short period results in a large additional burst of DNA synthesis at 39°C (Figure 8) seems most easily explained as a stimulation of DNA replication cycles. More direct tests to determine whether DNA molecules finish being replicated in FA21 at higher temperature will clarify these interpretations.

It is not clear whether the difference of behavior of the two mutants studied necessarily implies the existence of two distinct proteins involved in the synthesis of DNA. Although FA21 continues after transfer to higher temperature to make DNA at a rate initially identical to the wild type, while FA22 immediately achieves a rate slower than the wild type and characteristic of the higher temperature (Figure 2), the two could involve the same protein. In the case of FA22 one could assume that the sensitive enzyme *functions* less efficiently the higher the temperature. In the case of FA21 one could assume, for example, that the sensitive enzyme is fully active at all temperatures and that inactivation occurs only during function at higher temperature.

The observation that the phage λ, unlike T4, requires the temperature-sensitive function in the bacterial mutants for replication of its DNA may provide a convenient tool for probing the nature of the lesions. It will be of interest to determine the fate of the infecting λ DNA molecule and of λ prophage in the mutants at higher temperature. More insight into the nature of the lesions should come also from further investigation of the genetics of these mutants.

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SUMMARY

A procedure for the isolation of mutants of *Escherichia coli* with temperature-sensitive synthesis of DNA is reported, and characterization of two mutants typical of those most frequently encountered is presented. In one mutant transfer to higher temperature leads to an immediate fall in the rate of DNA synthesis; in the other, synthesis continues at a constant rate for approximately one hour before stopping. In both mutants, the effect on DNA synthesis is very preferential since RNA and protein synthesis continue for as much as a twenty-fold increase. After arrest of DNA synthesis, continuation of RNA and protein synthesis leads to rapid loss in viability. The block in DNA synthesis appears to affect replication directly since the deoxyribonucleoside triphosphate precursors of DNA can be made. Growth of phage T4 can occur but not of phage λ, indicating that the temperature-sensitive element in the bacteria is needed for normal λ development. In the mutant in which DNA synthesis continues for a time before stopping, the temperature-sensitive element does not appear to have the properties expected for the initiator of DNA synthesis postulated by others.

LITERATURE CITED

APIRION, D., 1966 Altered ribosomes in a suppressor strain of *Escherichia coli*. J. Mol. Biol. **16**: 285-301.

BONHOEFFER, F., 1966 DNA transfer and DNA synthesis during bacterial conjugation. Z. Vererb. **98**: 141-199.

EDLIN, G., and O. MAALØE, 1965 Synthesis and breakdown of messenger RNA without protein synthesis. J. Mol. Biol. **15**: 428-434.

EIDLIC, C., and F. C. NEIDHARDT, 1965 Protein and nucleic acid synthesis in two mutants of *Escherichia coli* with temperature-sensitive aminoacyl ribonucleic acid synthetases. J. Bacteriol. **89**: 706-711.

EPSTEIN, R. H., BOLLE, C. M. STEINBERG, E. KELLENBERGER, E. BOY DE LA TOUR, R. CHEVALLEY, R. S. EDGAR, M. SUSMAN, G. H. DENHARDT, and A. LIELAUSIS, 1963 Physiological studies of conditional lethal mutants of bacteriophage T4D. Cold Spring Harbor Symp. Quant. Biol. **28**: 375-392.

FANGMAN, W. L., 1966 Temperature-sensitive DNA synthesis in mutants of *Escherichia coli*. Bacteriol. Proc. p. 81.

FANGMAN, W. L., and A. NOVICK, 1966 Mutant bacteria showing efficient utilization of thymidine. J. Bacteriol. **91**: 2390-2391.

GILES, W. K., and A. MYERS, 1965 An improved diphenylamine method for the estimation of deoxyribonucleic acid. Nature **206**: 93.

HORIUCHI, T., J. TOMIZAWA, and A. NOVICK, 1962 Isolation and properties of bacteria capable of high rates of β-galactosidase synthesis. Biochim. Biophys. Acta **55**: 152-163.

JACOB, F., S. BRENNER, and F. CUZIN, 1963 On the regulation of DNA replication in bacteria. Cold Spring Harbor Symp. Quant. Biol. **28**: 329–347.

JACOB, F., and E. L. WOLLMAN, 1961 *Sexuality and the Genetics of Bacteria.* Academic Press, New York.

KOHIYAMA, M., D. COUSIN, A. RYTER, and F. JACOB, 1966 Mutants thermosensibles d'*Escherichia coli* K12. I. Isolement et caractérisation rapide. Ann. Inst. Pasteur (Paris) **110**: 465–486.

KOHIYAMA, M., H. LAMFROM, S. BRENNER, and F. JACOB, 1963 Modifications de fonctions indispensables chez des mutants thermosensibles d'*Escherichia coli*. Sur une mutation empêchant la réplication du chromosome bactérien. Compt. Rend. Acad. Sci. **257**: 1979–1981.

LARK, K. G., 1966 Regulation of chromosome replication and segregation in bacteria. Bacteriol. Rev. **30**: 3–32.

LOW, B., 1965 Low recombination frequency for markers very near the origin in conjugation in *E. coli*. Genet. Res., **6**: 469–473.

LOWRY, O. H., N. J. ROSEBROUGH, A. L. FARR, and R. J. RANDALL, 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193**: 265–275.

MAALØE, O., 1963 Role of protein synthesis in the DNA replication cycle in bacteria. J. Cellular Comp. Physiol., Suppl. 1, **62**: 31–44.

MAALØE, O., and N. O. KJELDGAARD, 1966 In *Control of Macromolecular Synthesis.* Benjamin, New York.

MENDELSON, N. H., and J. D. GROSS, 1967 Characterization of a temperature-sensitive mutant of *Bacillus subtilis* defective in deoxyribonucleic acid replication. J. Bacteriol. **94**: 1603–1608.

NEIDHARDT, F. C., 1966 Roles of amino acid activating enzymes in cellular physiology. Bacteriol. Rev. **30**: 701–719.

NEUHARD, J., and A. MUNCH-PETERSEN, 1966 Studies on the acid-soluble nucleotide pool in thymine-requiring mutants of *Escherichia coli* during thymine starvation. II. Changes in amounts of deoxycytidine triphosphate and deoxyadenosine triphosphate in *Escherichia coli* $15T^{-}A^{-}U^{-}$. Biochim. Biophys. Acta. **114**: 61–71.

RANDERATH, K., and E. RANDERATH, 1966 Ion exchange thin-layer chromatography. XV. Preparation, properties, and applications of paper-like PEI-cellulose sheets. J. Chromatography **22**: 110–117.

RICHARDSON, C. C., C. L. SCHILDKRAUT, H. V. APOSHIAN, and A. KORNBERG, 1964 Enzymatic synthesis of deoxyribonucleic acid. XIV. Further purification and properties of deoxyribonucleic acid polymerase from *Escherichia coli*. J. Biol. Chem. **239**: 222–232.

SADLER, J. R., and A. NOVICK, 1965 The properties of repressor and the kinetics of its action. J. Mol. Biol. **12**: 305–327.

SCHNEIDER, W. C., 1945 Phosphorous compounds in animal tissues. I. Extraction and estimation of desoxypentose nucleic acid and of pentose nucleic acid. J. Biol. Chem. **161**: 293–303.

SÉCHAUD, J., G. STREISINGER, J. EMRICH, J. NEWTON, H. LANFORD, H. REINHOLD, and M. M. STAHL, 1965 Chromosome structure in phage T4. II. Terminal redundancy and heterozygosis. Proc. Natl. Acad. Sci. U.S. **54**: 1333–1345.

TAYLOR, A. L., and M. S. THOMAN, 1964 The genetic map of *Escherichia coli* K12. Genetics **50**: 659–677.