EFFECTS OF PRIOR ALTERATION IN NUCLEIC ACID AND PROTEIN METABOLISM ON SUBSEQUENT MACROMOLECULAR SYNTHESIS BY IRRADIATED BACTERIA

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A previous communication (Billen, 1959*a*) described the alteration of the killing effect of X rays on *Escherichia coli* resulting from prior unbalanced growth. The possibility that the treatments by which the synthesis of protein, deoxyribonucleic acid (DNA), and ribonucleic acid (RNA) could be separated, would be reflected in altered postirradiation synthesis was next investigated.

A preliminary report (Billen, 1959*b*) suggested that the DNA synthesizing mechanism of bacteria, like that of animal cells, was composed of a radiosensitive and radioresistant system. These results plus the data presented here show that postirradiation synthesis of nucleic acids is markedly influenced by prior alteration in the macromolecular constituents of the organism.

MATERIALS AND METHODS

Bacterial strains and conditions of growth. *E. coli* strains B/r and 15_T- have been carried as stock cultures for two years. *E. coli* strain 15_T- was kindly provided, originally, by Dr. S. S. Cohen. At intervals this strain was checked for its thymine requirement. *E. coli* strain 15_T-- (555-7), requiring, in addition to thymine, three amino acids (arginine, tryptophan, and methionine), was generously provided by Dr. O. E. Landman. During routine maintenance this strain was found to exhibit variation into smooth and mucoid colony types. Both types were auxotrophic for the nutrients already listed. For the experiments reported here the smooth type was used because of the desirability of its plating characteristics.

The details of cell growth and composition of the minimal-salts medium have been described (Billen, 1959*a*, *b*). For *E. coli* strain 15_T-(555-7) the minimal medium plus thymine (20 μg/ml) was supplemented as follows: arginine, 380 μg/ml; tryptophan, 140 μg/ml; methionine, 300 μg/ml. In determining the number of colony-forming cells, agar plates of similar composition to that of the liquid media were employed. Incubation for 24 hr at 37 C was sufficient for maximal colony formation.

Log phase cells were used in all experiments. The chloramphenicol level was 10 μg/ml where added.

Irradiation and chemical analysis. After the treatments to be described, harvested cells were washed once in 50 per cent of the original volume of minimal medium without supplements, concentrated 10 to 20 times (1 to 5 $\times$ 10^9 cells per ml) over the original culture and exposed to X rays as previously described (Billen, 1959*a*). These steps were carried out at ice-bath temperatures. After X-ray exposure, the cells were added to twice the original culture volume of warmed minimal medium containing the necessary supplements for maximal growth and incubated for study of their metabolic capacity. At the indicated intervals, 50 ml samples of culture were removed for assay. DNA, RNA, and protein determinations were carried out by the modified Schmidt and Thannhouser procedure (*see* Billen, 1959*b*).

RESULTS

Synthesis of nucleic acid and protein by irradiated log phase cells. The initial rate of synthesis of DNA, RNA, and protein by washed (nontreated) log phase *E. coli* strain 15_T- exposed to 10,000 r was only partially reduced as compared to unirradiated controls (figures 1–3, no pretreatment). Some cell lysis apparently occurred at about 60 min postirradiation growth, since a reduction in turbidity as well as a decrease in all three constituents was noted during the following 20 to 60 min. Beyond this time renewed synthesis was observed in the irradiated cultures, presumably reflecting growth of the survivors. In

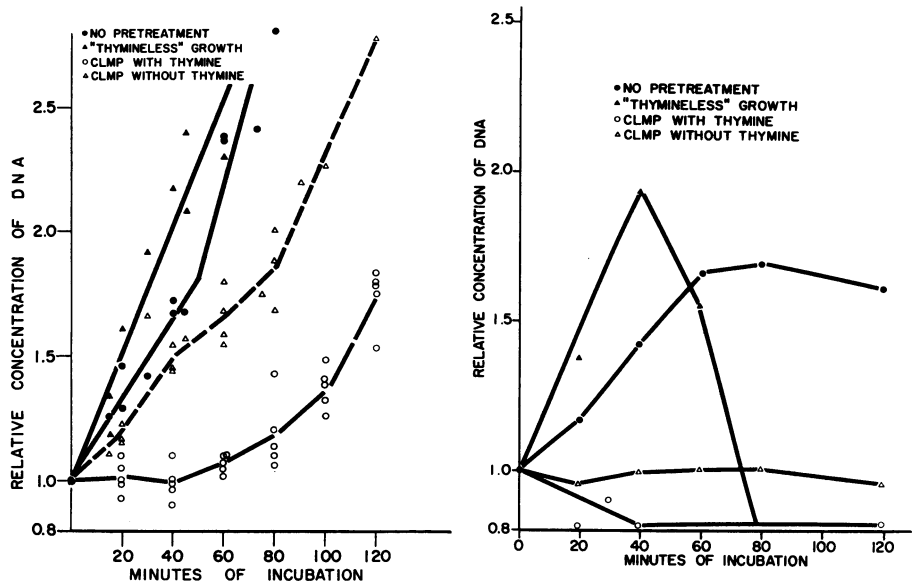

Figure 1. Synthesis of deoxyribonucleic acid (DNA) in *Escherichia coli* strain 15_T- after unbalanced growth. *A, left.* Controls: Cells incubated in minimal-salts medium plus thymine. *B, right.* Cells exposed to 10,000 r (X rays) before incubation in minimal-salts medium plus thymine. Values for the exposed cells represent the mean of two or more experiments. Details of the pretreatments are described in the text. *CLMP* = chloramphenicol.

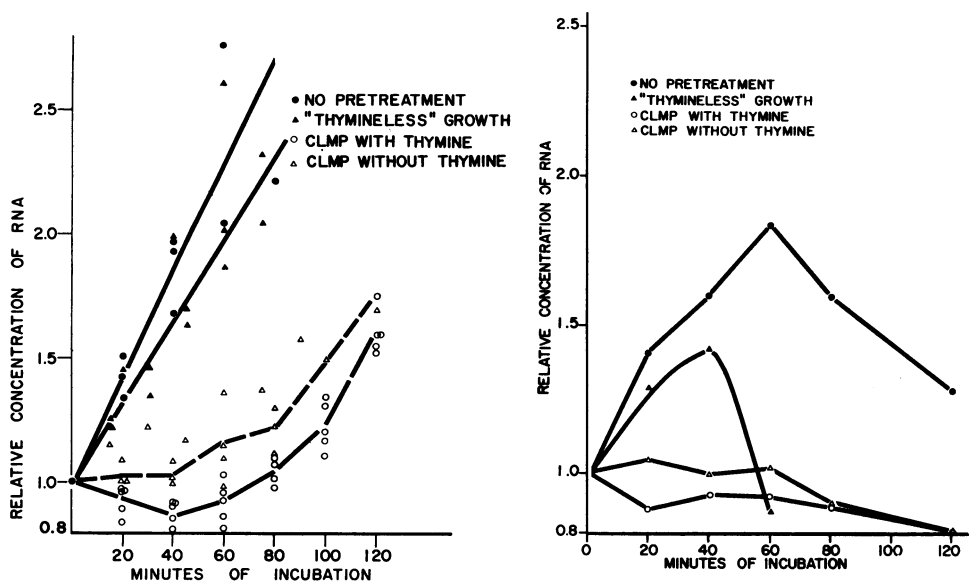

Figure 2. Synthesis of ribonucleic acid (RNA) in *Escherichia coli* strain 15_T- after unbalanced growth. *A, right.* Control. *B, left.* Exposed. Conditions as described in figure 1. *CLMP* = chloramphenicol.

the irradiated cell suspensions a division delay occurred: the number of viable cells remained constant or decreased to a varying extent during the first 40 to 60 min of culture (table 1). By 60 min the number of colony-forming cells began to increase.

Effects of chloramphenicol pretreatment on subsequent synthesis. When chloramphenicol is added

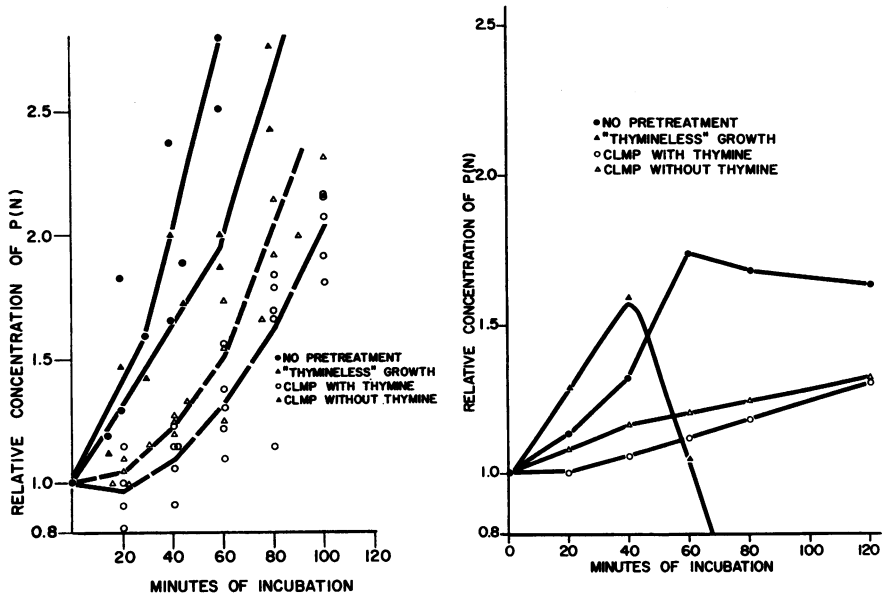

Figure 3. Synthesis of protein (*PN*) in *Escherichia coli* strain 15_T- after unbalanced growth. *A, right.* Control. *B, left.* Exposed. Conditions as described in figure 1. *CLMP* = chloramphenicol.

TABLE 1

Effects of pretreatment on cell division and survival

Pretreatment	Cell Titer/ml During Exposure	Minutes to Beginning of Division		Surviving Fraction (10,000 r)
		Control	10,000 r	
1. None				
Exp. 1106	3.8×10^9	10–20	50–60	1.3×10^{-1}
1110	8.2×10^9	0–10	40–60	4.7×10^{-1}
1121	5.7×10^9	0–20	40–60	2.9×10^{-1}
1132	5.8×10^9	0–15	60–75	8.4×10^{-2}
2. Presence of chloramphenicol				
Exp. 1104	4.3×10^9	40–50	90–100	6.6×10^{-1}
1112	3.5×10^9	60–90	>120	5.9×10^{-1}
1113	2.6×10^9	60–90	90–120	3.2×10^{-1}
3. Thymine starved				
Exp. 1129	1.9×10^9	30–60	60–90	2.5×10^{-1}
1134	4.0×10^9	0–15	45–60	6.2×10^{-2}
1170	1.5×10^9	20–40	60–80	7.0×10^{-2}
4. Thymine starved in presence of chloramphenical				
Exp. 1118	3.0×10^9	60–90	60–90	1.2×10^{-1}
1119	2.0×10^9	60–80	80–100	1.3×10^{-1}
1133	4.2×10^9	40–60	60–80	8.3×10^{-3}
1169	2.0×10^9	40–60	80–120	5.6×10^{-3}

to cultures of log phase *E. coli*, protein synthesis is severely inhibited (Wisseman *et al.*, 1954). The unbalanced growth taking place during incubation of *E. coli* strain 15_T- for 1 hr in the presence of chloramphenicol has been described (Billen, 1959*a*). When cells so treated were washed free of chloramphenicol they showed an altered metabolic capacity. The synthesis of DNA and RNA by these cells was delayed about 30 and 60 min, respectively, when they were reincubated at

37 C in growth medium (figures 1*A*, 2*A*, pretreatment: chloramphenicol with thymine). In contrast to studies (Hahn *et al.*, 1957; Neidhardt and Gros, 1957), on other strains of *E. coli*, net loss of nucleic acid from the cells during this period was relatively slight. This lag in synthesis was followed by a rapid increase in both nucleic acids.

Thirty to seventy per cent killing was observed in chloramphenicol treated cells exposed to 10,000 r. Upon incubation such cells were unable to recover their capacity to synthesize nucleic acids (figures 1*B*, 2*B*, pretreatment: chloramphenicol with thymine) during the 2 hr observation. Protein synthesis was also affected in such irradiated cells although a net synthesis was usually observed (figure 3*B*). Division delays were most pronounced among the antibiotic pretreated, exposed survivors (table 1).

Additional experiments showed that the post-irradiation (10,000 r) addition of chloramphenicol to log phase cells not previously treated failed to prevent DNA and RNA synthesis by such cells. There was some decrease in rate, but no more than might be expected from the effects of chloramphenicol on unirradiated log phase cells.

Influence of DNA synthesis during chloramphenicol treatment. Barner and Cohen (1954) showed that in the absence of exogenous thymine, DNA synthesis in *E. coli* strain 15_T- was suppressed. Upon addition of thymine a synchronization of the synthesis of DNA was observed. We obtained similar results following thymine starvation for 60 min (Billen, 1959*a*, *b*). Upon subsequent addition of thymine the bacteria synthesized DNA at a more rapid rate than did log phase cells. In the experiment discussed here, 30 min of thymine starvation was used to avoid thymineless death. Although there was no thymineless death during the 30 min of thymine deprivation, such cells were more radiosensitive (Billen, 1959*a*). After exposure to 10,000 r, DNA synthesis by such cells was initially little altered in rate and the amount of DNA approximately doubled, whereas protein and, to a greater extent, RNA synthesis, were more strongly depressed (figures 1*B*, 2*B*, 3*B*, pretreatment: thymineless growth). Marked lysis was characteristic of such exposed cell cultures by 40 to 60 min as evidenced by a fall in turbidity and a more drastic decrease in cellular constituents, including protein.

If chloramphenicol is present in the culture medium from which thymine has been omitted, only RNA will accumulate in large quantity (Billen, 1959*a*). When chloramphenicol is removed after incubation for 1 hr without thymine, and the cells are incubated in growth medium, there is approximately the same lag as that found for chloramphenicol-treated log phase cells in the presence of thymine; however, DNA synthesis commences almost immediately (figures 1*A*, 2*A*, 3*A*, pretreatment: chloramphenicol without thymine).

Exposure of these cells to 10,000 r again resulted in complete inhibition of DNA and RNA synthesis (figures 1*B*, 2*B*, pretreatment: chloramphenicol without thymine). Protein synthesis occurred but was limited (figure 3*B*). In both controls and exposed suspensions an extended delay in division was seen, being more pronounced for the irradiated survivors (table 1). The severe lysis seen in irradiated cells prestarved of thymine was not observed in these cells that had been starved in the presence of chloramphenicol.

Effects of prior amino acid starvation on post-irradiation synthesis in an E. coli strain 15_T- polyauxotroph. Protein synthesis was prevented in the multiple amino acid requiring auxotroph *E. coli* strain 15_T-(555-7) when washed cells were incubated for 90 min at 37 C in an amino acid-free minimal medium supplemented with thymine. Protein and RNA synthesis was completely halted after an initial synthesis during the first 30 min. DNA synthesis was less affected and continued at a reduced rate during this time. The cells were able to complete one additional division. Thus during this starvation period, protein and RNA synthesis did not occur during the final 60 min of culture. These findings on RNA, DNA, and protein metabolism during amino acid starvation are similar to those reported by Barner and Cohen (1957) in their studies with other mutants of strain 15_T- requiring amino acids.

Exposure of such cells to 10,000 r caused a marked inhibition of initial DNA synthesis when the cells were incubated in a supplemented medium (figure 4). RNA and protein synthesis did not show this delay but proceeded at a reduced rate (figures 5 and 6). Although division by the viable cells in the irradiated population was again delayed for approximately 60 min (the unirradiated controls showed a division delay of approximately 30 min), the large number of sur-

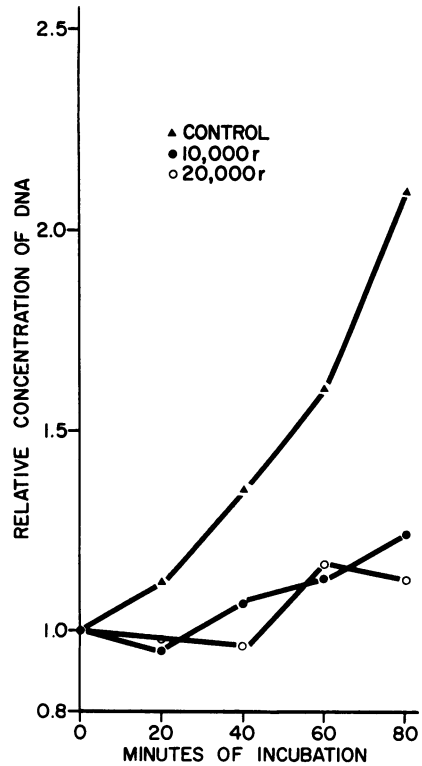

Figure 4. Synthesis of deoxyribonucleic acid (DNA) after incubation of *Escherichia coli* strain 15_T (555-7) in amino acid deficient minimal-salts, thymine medium. After irradiation the cells were incubated in minimal-salts medium supplemented with thymine and the required amino acids. The values presented represent the mean of at least three experiments. Details of the experimental procedure are given in the text.

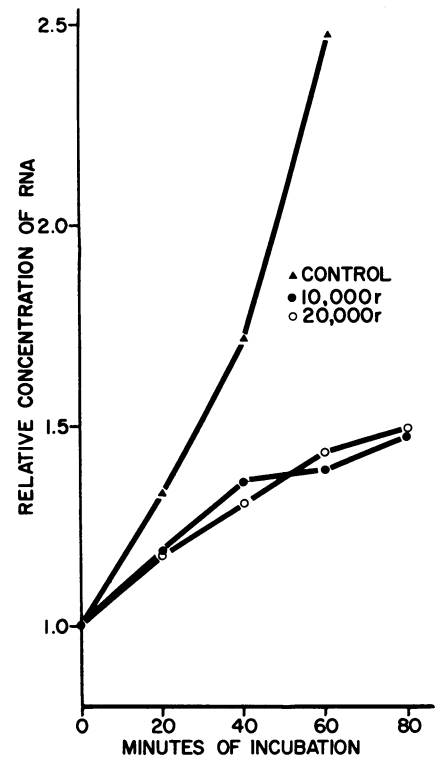

Figure 5. Synthesis of ribonucleic acid (RNA) after incubation of *Escherichia coli* strain 15_T–(555-7) in amino acid deficient minimal-salts, thymine medium. Conditions as described in figure 4.

vivors (25 to 35 per cent) made it difficult to evaluate the true extent of the metabolic inhibition on the affected cells. When a dose of 20,000 r was delivered to suspensions similarly pretreated, approximately 5 per cent of the cells survived. In this experiment the contribution of the survivors would be negligible (an 80-min lag before increase in cell number was also observed). DNA synthesis was found to be completely prevented for the first 40 min (figure 4). RNA synthesis was much like that found in cells exposed to 10,000 r (figure 5) whereas protein synthesis was more severely depressed (figure 6). Log phase (non-starved), irradiated (10,000 r) *E. coli* strain 15_T–(555-7) did not show this extended lag in DNA synthesis (figure 7).

Treatment with p-fluorophenylalanine. To determine whether the formation of "abnormal" proteins would interfere with the establishment of the radioresistant DNA synthesizing system, *E. coli* strain 15_T– was exposed to *p*-fluorophenylalanine during log growth. Enough *p*-fluorophenylalanine was added to give a final concentration of 1×10^{-3} M and the incubation continued for 90 to 120 min. Although direct detection of the incorporation of the analogue into cellular protein was not attempted, the conditions of culture were similar to those reported by others (Cohen and Munier, 1959; Gros and Gros, 1958) to result in large scale incorporation of the analogue by *E. coli*. In our investigation the addition of the analogue to log phase cultures of *E. coli* strain 15_T– caused a shift to linear growth, based on dry weight measurements, within 30 min. Cell division had ceased by 60 min.

When organisms were exposed to 10,000 r or 15,000 r, the synthesis of DNA, RNA, and pro-

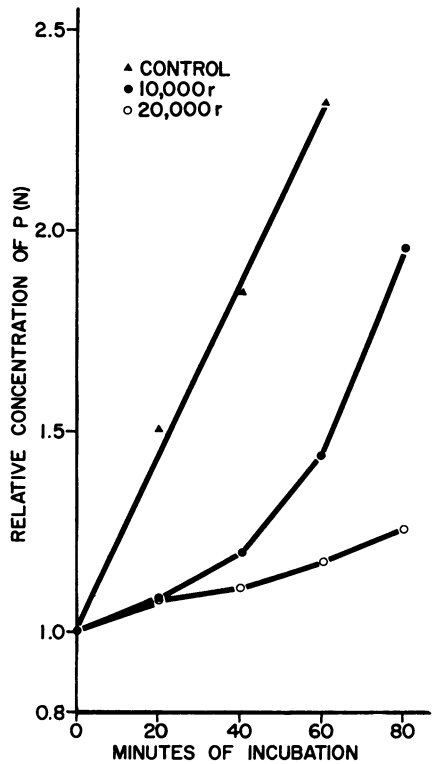

Figure 6. Synthesis of protein [$P(N)$] after incubation of *Escherichia coli* strain 15_T-(555-7) in amino acid deficient minimal-salts, thymine medium. Conditions as described in figure 4.

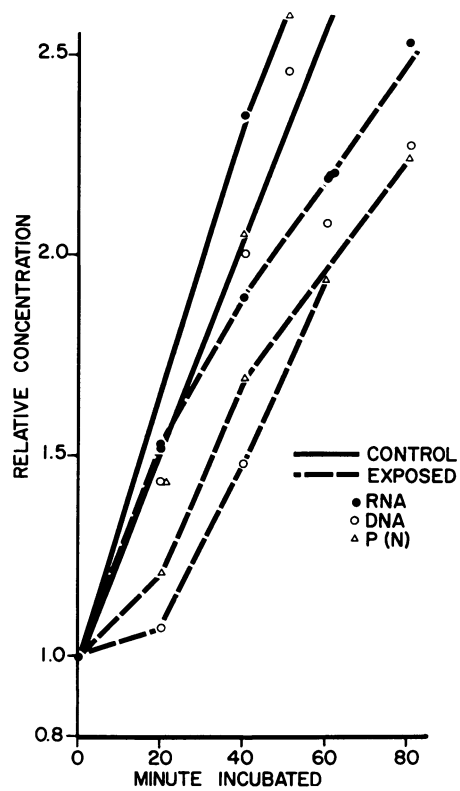

Figure 7. Synthesis of nucleic acid and protein [$P(N)$] in log phase cultures of *Escherichia coli* strain 15_T-(555-7) exposed to 10,000 r. After exposure the cells were incubated in minimal-salts medium supplemented with thymine and the required amino acids (methionine, arginine, and tryptophan).

tein was little affected compared to the controls. These cells responded in a manner similar to log phase cells (no pretreatment).

Influence of chloramphenicol pretreatment on macromolecular synthesis on irradiated E. coli strain B/r. To determine whether the modification in radiation response brought about by interference in protein synthesis was restricted to *E. coli* strain 15_T- alone, *E. coli* strain B/r was subjected to the chloramphenicol pretreatment before X-irradiation. Log phase cells of *E. coli* strain B/r were found to show a prolonged delay in nucleic acid synthesis after exposure to the antibiotic (figure 8). Synthesis of RNA and DNA was delayed approximately 90 min. A decrease in intracellular RNA was usually seen during this period in confirmation of the results of Hahn *et al.* (1957) in their studies on *E. coli* strain B/r. At about 2 hr posttreatment, growth was renewed and by 3 hr the DNA, RNA, and protein content had at least doubled. A dose of 10,000 r prevented the recovery of DNA synthesis by such cells for at least the 3 hr of observation (figure 8). In fact, a loss of cellular DNA was observed during this period. A loss of RNA at a rate similar to that in the unirradiated cells was observed; however, renewed synthesis had not begun after 3 hr in the exposed cells.

Log phase cultures of *E. coli* strain B/r (no chloramphenicol pretreatment), after exposure to 10,000 r, were able to synthesize DNA, RNA, and protein at a rate similar to that illustrated for log phase *E. coli* strain 15_T-(555-7) cells following their exposure to 10,000 r. For example, at 60 min postirradiation incubation, the DNA, RNA, and protein content of such cultures increased 64, 81, and 87 per cent, respectively. As with *E. coli* strain 15_T-(555-7), no lysis was

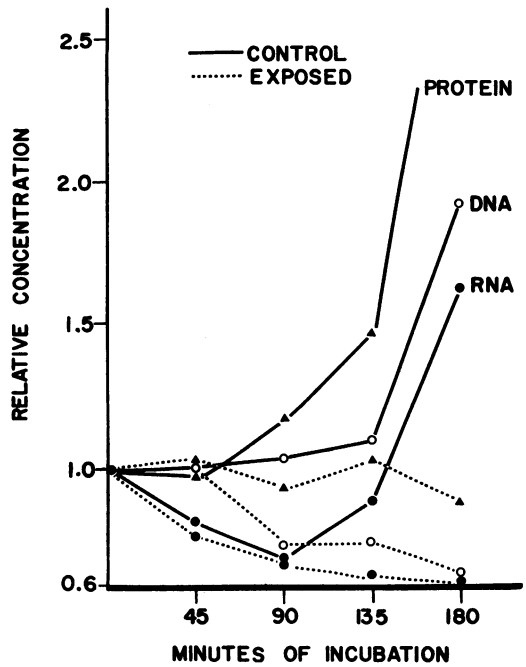

Figure 8. Synthesis of nucleic acid and protein in *Escherichia coli* strain B/r incubated with chloramphenicol. After exposure to chloramphenicol for 1 hr a sample of the washed cells was exposed to 10,000 r. The cells were then incubated in minimal-salts medium.

detectable in the irradiated cultures of *E. coli* strain B/r.

DISCUSSION

The results of this work make it obvious that specific alterations in the physiological activities of a bacterial population will markedly influence its postirradiation capacity for macromolecular synthesis. With respect to DNA synthesis, the results with bacteria are somewhat similar to those reported for mammalian cell studies. The DNA synthesizing capacity of irradiated animal cells is closely related to the stage in their division cycle during which they are exposed (*see* Howard, 1956; Lajtha *et al.*, 1958*a*). For example, Caspersson, Klein, and Ringertz (1958) concluded from their studies with ascites tumors that "the cells which contained the double DNA content when they were irradiated are obviously unable to synthesize more DNA, while those which have not yet begun or just started their synthesis of DNA are able to reach the double DNA value." These workers suggest that damage to the mitotic process may be the primary effect involved in blockage of further DNA synthesis. Other investigators have separated the synthetic period of DNA synthesis (S) from the presynthetic period (G_1) by observing the highly radiosensitive response of the latter (Howard and Pelc, 1953; Lajtha *et al.*, 1958*a*). The S period has been further separated into an S_1 and S_2 component system, the latter being extremely radioresistant (Lajtha *et al.*, 1958*b*). The G_1 and S_1 period may be concerned with precursor synthesis, liberation of inhibitory enzyme systems, i. e., deoxyribonucleases, uncoupled oxidative phosphorylation or any of a number of possibilities related to cell metabolism or structure (Ord and Stocken, 1958). Whatever the mechanism involved, most observations suggest that it is concerned only with DNA synthesis, since RNA and protein synthesis are not always found to be greatly inhibited. It was suggested that the S_2 component represents the integrity of the DNA template (Lajtha *et al.*, 1958*b*). The synthesis of DNA in large quantity in certain mammalian cells (giants) studied *in vitro* (Whitmore *et al.*, 1958) suggests that the final interpretation may be more complicated.

In our own studies on bacterial cells a similar separation of the DNA-synthesizing mechanism into a pre-DNA-synthetic system, which is radiosensitive, and a more radioresistant DNA-synthesizing system appears possible by controlling the extent of protein synthesis prior to exposure. This has been discussed in a preliminary report (Billen, 1959*b*). The nature of the proteins involved in the presynthetic system is unknown. This may be concerned with the metabolic and structural changes already mentioned above in accounting for the radiosensitivity of the G_1 period in animal cells. For example, there is the possibility that specific nucleic acid precursors may not be available due to reversible inhibition of the formation of necessary enzymes by blocking of protein synthesis during the pretreatment. The subsequent irradiation would irreversibly inhibit synthesis of these enzymes and thus preclude DNA synthesis. Yeast extract and peptone have been found to have no effect when added during the postirradiation period to chloramphenicol treated cells (Billen, *unpublished observations*). The effects of postirradiation addition of deoxyribonucleosides and other materials are now under investigation. The possibility that

the pretreatments have led to depletion of the polymerase enzyme necessary for DNA synthesis (Lehman *et al.*, 1958) and that the formation of polymerase is radiosensitive, whereas polymerase activity is relatively radioresistant, will also be considered in future studies.

If indeed the synthesis of protein is involved in the development of a normal DNA-synthesizing system, the failure of pretreatment with *p*-fluorophenylalanine to influence DNA synthesis following irradiation is puzzling in light of the reports on its incorporation into cellular protein. One might speculate that the analogue is not equally incorporated into all proteins made by the cell or that its incorporation into certain proteins does not interfere with their activity.

Our results do not exclude a role for RNA in the pre-DNA-synthetic system since the RNA formed in the presence of chloramphenicol may be biologically inactive. In addition, amino acid starvation of the auxotroph used in our studies not only brings protein synthesis to a halt, but also stops net RNA synthesis.

Interpretation of the results in chloramphenicol-pretreated cells might be complicated by the presence of residual antibiotic in the washed cells which could influence subsequent synthesis. This is unlikely for the following reasons: (a) The incubation of irradiated log phase cells, in growth medium containing chloramphenicol did not prevent RNA and DNA synthesis. (b) In cells of *E. coli* strain 15_T-, pretreated by exposure to chloramphenicol in the absence of thymine (no DNA synthesis), there was rapid DNA synthesis upon subsequent incubation in the presence of thymine. It was only under conditions of DNA surplus, i. e., chloramphenicol exposure in the presence of thymine, that DNA synthesis was delayed upon reincubation following removal of the antibiotic. Thus the delay in nucleic acid synthesis observed following chloramphenicol exposure seems to be dependent on the quantity of surplus nucleic acid accumulated. The observation that irradiation completely suppressed DNA synthesis in cells that had been preincubated with chloramphenicol independently of the presence or absence of thymine suggests the following interpretation. There is a labile protein component necessary for DNA synthesis. The loss of this protein is not dependent on DNA synthesis since in the presence of chloramphenicol and the absence of thymine, it disappears. Removal of the chloramphenicol allows renewed synthesis of this substance; X-ray exposure prevents renewal.

These results, suggesting the involvement of protein in the mechanism of X-ray damage to the DNA-synthesizing system, are not unlike the findings of Harold and Ziporin (1958), Doudney (1959), and Drakulic and Errera (1959), that synthesis of RNA and protein appears to be necessary for repair of a block in DNA synthesis induced by ultraviolet light. Whereas exposure of log phase *E. coli* to ultraviolet causes an immediate but reversible inhibition of DNA synthesis, exposure to X rays brings about a different response: synthesis starts immediately, albeit at a reduced rate which is dependent on dose. From the information now available it appears that, whereas ultraviolet destroys or temporarily inhibits the activity of the actual DNA synthesizing component (template, polymerase activity, etc.), ionizing radiation acts initially by halting synthesis of those components (protein and RNA) necessary for final DNA replication. Once the components making up the DNA synthetic system have been formed, higher doses of ionizing radiation are necessary for halting the final steps in DNA synthesis.

From the results presented it is clear that RNA and protein synthesis are also severely affected by the pretreatments. Following growth in the presence of chloramphenicol, RNA synthesis was as completely inhibited by irradiation as was DNA synthesis. However, following amino acid starvation in the auxotroph, RNA synthesis was partially refractory to subsequent irradiation while DNA synthesis was completely prevented for a period of time. In the former experiment an RNA surplus existed at the time of exposure, in the latter experiment RNA as well as protein synthesis was prevented during the pretreatment. These results are somewhat reminiscent of the findings of Borek, Rochenback, and Ryan (1956) with a mutant of *E. coli* requiring methionine that accumulated RNA in the absence of methionine. These investigators found that new RNA synthesis by methionine-starved cells was inhibited after ultraviolet exposure. In our studies also those cells accumulating abnormally high quantities of RNA through prior unbalanced growth were observed to show a greater retardation in RNA synthesis following irradiation.

An explanation of the lysis induced by X rays

in *E. coli* strain 15_T- is lacking. It is clear that starving the cells for thymine prior to exposure enhances the induction of lysis by X rays. A preliminary check on the possible release of agents lytic for *E. coli* strain B from log phase *E. coli* strain 15_T- exposed to 10,000 r has produced negative results (Humphrey and Billen, *unpublished data*). It is of interest to point out that the polyauxotroph strain 15_T- (555-7), derived from strain 15_T-, did not lyse following X-ray exposure. It should also be noted that prior exposure to chloramphenicol greatly modified lysis induction.

Similar observations have been made on these strains following ultraviolet light exposure (Weatherwax and Landman, *personal communications*).

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SUMMARY

Synthesis of nucleic acids and protein was partially inhibited after exposure of log phase *Escherichia coli* strain 15_T- (thymine requiring) and strain B/r to 10,000 r of X rays.

Incubation of log phase *E. coli* strains 15_T- and B/r with chloramphenicol altered the radiosensitivity of the macromolecular synthesizing systems of these cells. Reversal of the chloramphenicol induced lag in the synthesis of nucleic acid was prevented in such cells exposed to 10,000 r. Protein synthesis was considerably reduced.

Incubation of *E. coli* strain 15_T- in the absence of thymine did not adversely influence their initial nucleic acid synthesizing capacity following a dose of 10,000 r.

When chloramphenicol was added to *E. coli* strain 15_T- undergoing thymineless growth, both deoxyribonucleic acid (DNA) and protein synthesis were prevented. Irradiation prevented resumption of nucleic acid synthesis upon incubation of these cells in thymine-supplemented minimal medium. Unirradiated cultures did not exhibit a delay in DNA synthesis, presumably due to the absence of thymine during the pretreatment with chloramphenicol.

Growth of the polyauxotroph, *E. coli* strain 15_T- (555-7) in the absence of essential amino acids prior to X-ray exposure increased the inhibitory effects of irradiation on macromolecular synthesis in these cells as compared to nonstarved log phase cells.

Growth of *E. coli* in the presence of the amino acid analogue, *p*-fluorophenylalanine, did not alter the effects of X-ray exposure on nucleic acid synthesis.

These results suggest that the synthesis of a protein constituent(s) is a necessary part of the presynthetic system in DNA replication. The presence of chloramphenicol or deprivation of essential amino acids apparently prevented the formation of this component. Upon removal of the block in protein synthesis there was a resumption in the production of the necessary constituent. X-ray exposure prevented the renewed synthesis of the protein needed for DNA replication.

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