The Effect of *p*-Fluorophenylalanine on Nucleic Acid Biosynthesis and Cell Division in *Escherichia coli**

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ABSTRACT: Cultures of *Escherichia coli* 15_T- were phased with respect to cell division by thymine starvation. *p*-Fluorophenylalanine blocked cell division and ribonucleic acid (RNA) polymerase synthesis when it was added to the culture at the same time as thymine. Addition of *p*-fluorophenylalanine (FPA) just prior to cell division (55 min after thymine) did not stop the division process for one step but inhibited RNA polymerase synthesis. FPA did not inhibit a rapid early synthesis of deoxyribonucleic acid (DNA) and isocitric dehydrogenase.

The results are best explained by assuming the FPA interferes with initiation of replication of DNA but allows completion of the cycle in progress. Similar interpretations have been used to explain the effect of amino acid starvation on bacterial cells.

When *Escherichia coli* B is treated with FPA[1] (10^{-3} M), the normal exponential rate of growth is slowed to a linear rate (Munier and Cohen, 1956). This linear phase is supplanted by a slow logarithmic phase which is followed by the rapid growth of a FPA-resistant mutant (Previc and Binkley, 1964a). During the linear growth phase, cell division stops almost completely, resulting in extensive filamentation.

The proposal has been made (Munier and Cohen, 1959) that since FPA is incorporated into protein it may render one or more enzymes nonfunctional. Various enzymes have been isolated and studied. Some have been found to show no altered activity (Cowie *et al.*, 1959; Vaughan and Steinberg, 1960; Yoshida, 1960), whereas the exopenicillinase of *B. cereus* (Richmond 1960) was found to be enzymically defective and immunologically altered. More recently studies of the effect of FPA on various viral systems (Zimmerman and Schafer, 1960; Ebisuzaki, 1963; Wilcox and Ginsberg, 1963) and on cultured mammalian cells (Lieberman *et al.*, 1963) indicate a direct influence of FPA on the appearance of activity of enzymes related to the synthesis of RNA and DNA.

The present study is an investigation of the effect of FPA on nucleic acid biosynthesis in *E. coli*. A survey of the changes in the rates of the synthesis of RNA and DNA in both logarithmically growing cultures and cultures which have been phased with respect to cell division has been made. A comparison of the appearance of activity of a respiratory enzyme, isocitric dehydrogenase, and of DNA-dependent RNA polymerase has been made under conditions of FPA inhibition. Also, the effect of the reversal of inhibition by tyrosine and phenylalanine has been studied with particular reference to changes in cell division.

Materials and Methods

Additives and Substrates. FPA was obtained from Calbiochem and from Sigma Chemical Co.; L-phenylalanine, ATP, CTP, UTP, and NADP from Sigma Chemical Co.; L-tyrosine from Eastman Kodak Co.; puromycin from Nutritional Biochemicals Corp.; uracil-2-^{14}C (0.5 mc/mmole), thymine-2-^{14}C (1.05 mc/mmole), and guanine-8-^{14}C (1.23 mc/mmole) from Volk Radiochemical Co.; and GTP and GTP-8-^{14}C (26 mc/mmole) from Schwarz BioResearch, Inc.

Cultures. E. coli B was maintained on nutrient agar slants in our laboratory. A culture of the mutant, *E. coli* 15_T-, was a gift from Dr. Seymour Cohen.

Growth. Cell cultures were grown in a modified Davis and Mingoli (1950) medium containing the following (grams per liter): K_2HPO_4 (7.0), KH_2PO_4 (3.0), trisodium citrate dihydrate (0.5), $(NH_4)_2SO_4$ (1.0), D-glucose (5.0), and 1.0 M $MgSO_4$ (0.5 ml/l.). Thymine (2 μg/ml) was added to the medium for the growth of the thymine-requiring mutant. Other additives were as indicated.

The cultures were incubated at 37° on a New Brunswick Model G-25 gyrotory shaker. Growth was followed turbidimetrically at 450 mμ on a Beckman DU spectrophotometer. Cell counts were made on a Coulter counter Model B. Samples of 1 ml from the cultures were added to 0.5 ml of 0.6% formaldehyde solution and aliquots were removed and diluted to 50 ml with isotonic saline for counting.

Cell division in cultures of *E. coli* 15_T- was phased by the following procedure; logarithmically growing cultures were filtered through a 0.45-μ Millipore filter

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[1] Abbreviations used: FPA, *p*-fluorophenylalanine; PA, phenylalanine; ATP, CTP, UTP, GTP, adenosine, cytidine, uridine, and guanosine triphosphates; NADP, nicotinamide-adenine dinucleotide phosphate; TCA, trichloroacetic acid; UDPAG, uridine diphosphoacetylglucosamine.

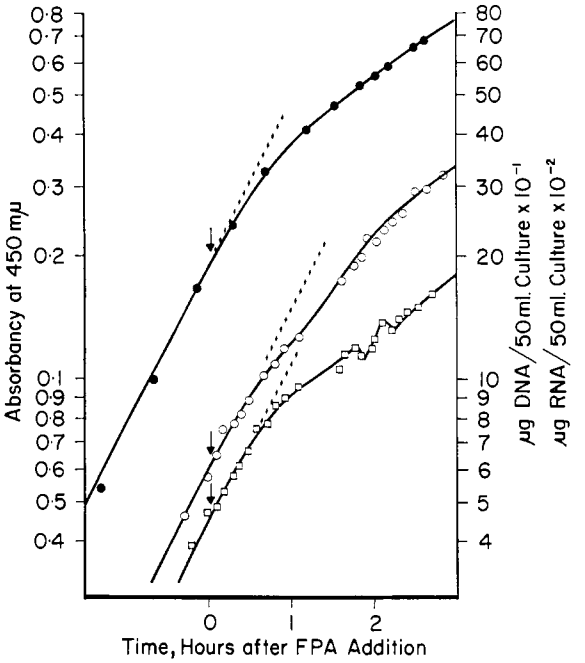

FIGURE 1: The relationships between RNA synthesis, DNA synthesis, and mass increase in the presence of FPA (10^{-3} M). (●) Absorbancy. (○) DNA. (□) RNA. (↓) Time of addition of FPA. (---------) Uninhibited culture. The variability in the synthesis of RNA in inhibited cultures at about 2 hr has been observed in many experiments.

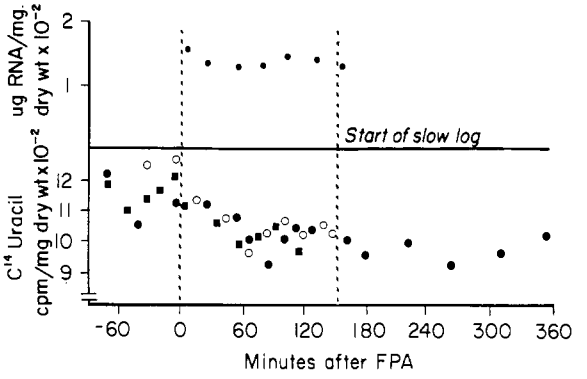

FIGURE 2: Comparison of RNA per milligram dry weight of cells. The upper graph was determined colorimetrically. The lower graph was determined by incorporation of uracil-^{14}C. ●, ○, and □ show results of three separate experiments. Incorporation of guanine-^{14}C gave the same results.

and the cells were washed with an equal volume of medium lacking glucose and thymine. The cells were resuspended in medium lacking thymine and incubated for 45 min. Thymine was added at the end of this incubation period. The entire washing procedure was carried out at 37° and required less than 5 min. It should be noted at this point that the cells were phased with respect to cell division. The system gave consistent results from experiment to experiment and no further interpretation is imposed as to the specific ordering of other events within the cell cycle.

RNA and DNA Determinations. Cell extracts of *E. coli* B were made by placing 50-ml samples of a culture in an equal volume of a cold 10% TCA solution. After standing for a minimum of 30 min, the cells were removed by centrifugation and washed twice with cold 5% TCA. The washed cells were extracted with 2 ml of 5% TCA at 70° for 30 min. The supernatant solution obtained from the hot extraction procedure was used for RNA and DNA determinations. RNA was determined by the orcinol method and DNA by the diphenylamine method as described by Panos *et al.* (1959).

Guanine-^{14}C, Uracil-^{14}C, and Thymine-^{14}C Incorporation. Guanine (sp act. 0.01 mc/mmole) was added to the medium to give a final concentration of 2.65×10^{-4} M and uracil (sp act. 0.005 mc/mmole) was added to give a final concentration of 5×10^{-4} M. Samples of 4 ml were removed from the culture at various intervals of time and added to an equal volume of cold 10% TCA. After standing for a minimum of 30 min, the precipitates were collected on 25-mm, 0.45-μ Millipore filters and washed with 5 ml of cold 3% TCA, and then with 5 ml of cold water. The filters were glued to aluminum planchets and counted in an end-window gas-flow counter. The same procedure was used when thymine (sp act. 0.17 mc/mmole) incorporation was followed.

Enzyme Assays. Aliquots of a culture were removed such that the total mass was relatively constant for all samples (32–36-ml absorbancy units). The samples were chilled rapidly and the cells obtained by centrifugation were washed once with cold complete medium, collected by centrifugation, and stored frozen overnight. After thawing, the cells were suspended in 2 ml of the buffer to be used in the enzyme assay, 0.02 ml of toluene was added, and the suspension was incubated for 20 min at 37° with gentle shaking. The extracts were chilled rapidly and clarified by centrifugation. The supernatant solution was used for the isocitric dehydrogenase assay. Isocitric dehydrogenase was determined by the method of Kornberg (1955). Assays were carried out using a Perkin-Elmer recording spectrophotometer.

RNA polymerase was assayed by a modification of the method of Hurwitz (1963). The reaction mixture consisted of the following: 0.05 ml of potassium maleate buffer (0.5 M), pH 7.5; 0.01 ml of 2-mercaptoethanol (0.2 M); 0.02 ml of $MnCl_2$ (0.1 M); 0.01 ml of [^{14}C]GTP (sp act. 0.55 mc/mmole); 0.05 ml of a solution of ATP, UTP, and CTP (0.0016 M with respect to each); 0.04 ml of calf thymus DNA (1 mg/ml); and 0.13 ml of cell extract. The reaction was incubated for 20 min at 38°, following which the tubes were placed in ice and 1 ml of 4% PP_i, 0.05 ml of bovine serum albumin (30 mg/ml), and 1.5 ml of cold 10% TCA were added. The precipitate was removed by centrifugation and suspended in 3 ml of cold 5% TCA. The sediment collected by centrifugation was again washed with 2.5 ml of cold 5% TCA, filtered on Whatman GF/C 2.4-cm glass filter paper,

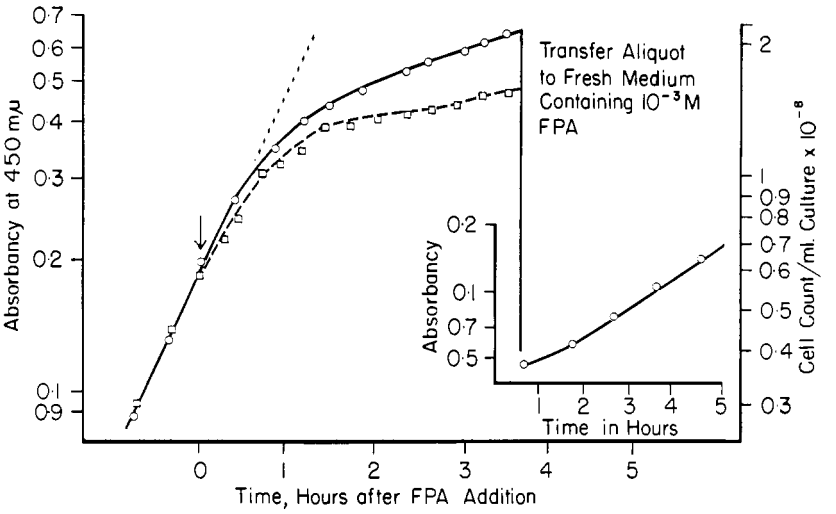

FIGURE 3: The effect of FPA (10^{-3} M) on the growth of *E. coli* 15_{T-}. (○) Absorbancy. (□) Cell counts.

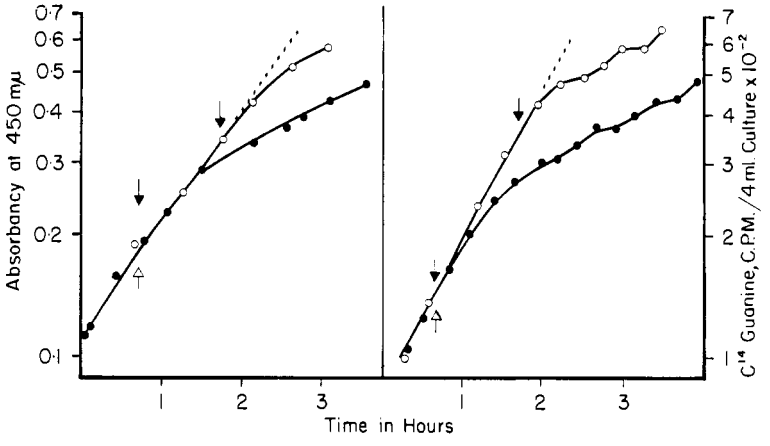

FIGURE 4: The effect of adding FPA (10^{-3} M) to phased cultures on increase in mass of the culture and the synthesis of RNA. (↑) Time of addition of thymine. (↓) Time of addition of FPA. (●) FPA added 1 min after thymine. (○) FPA added 55 min after thymine. (---------) Uninhibited culture. See Figure 5 for cell count.

and rinsed with 3 ml of cold 1% TCA and then 3 ml of ether. The filter papers were placed in small glass vials and dried at 60° for 2.5 hr, after which a toluene–phosphor solution was added. The vials containing the filters were placed in counting vials and the activity was determined in a Packard liquid scintillation counter.

Results

Effect of FPA on RNA and DNA Synthesis in E. coli B. The nucleic acid content of *E. coli* B growing in a glucose–salts medium increased in an exponential manner with time. When FPA was added to the cultures, the rates of RNA and DNA synthesis decreased. About 2 hr after addition of the analog, RNA and DNA began to increase exponentially, but at the reduced rate characteristic of slow log growth. The relationships between RNA and DNA synthesis and mass increase in the presence of FPA are shown in Figure 1. DNA and RNA were determined colorimetrically.

The course of RNA synthesis during FPA inhibition was also followed by the incorporation of guanine-^{14}C and uracil-^{14}C into the cold TCA-insoluble fraction of the cells. As observed when determinations were made colorimetrically, RNA synthesis became approximately linear after addition of the analog. Following the linear phase, a slow exponential phase was observed, the doubling time being two to three times longer than that of the untreated cultures. Upon transfer of an aliquot of a culture in slow exponential growth to fresh medium containing FPA and one of the labeled compounds, both growth and incorporation rates continued at the rate established just prior to transfer.

A comparison of the amount of RNA per milligram

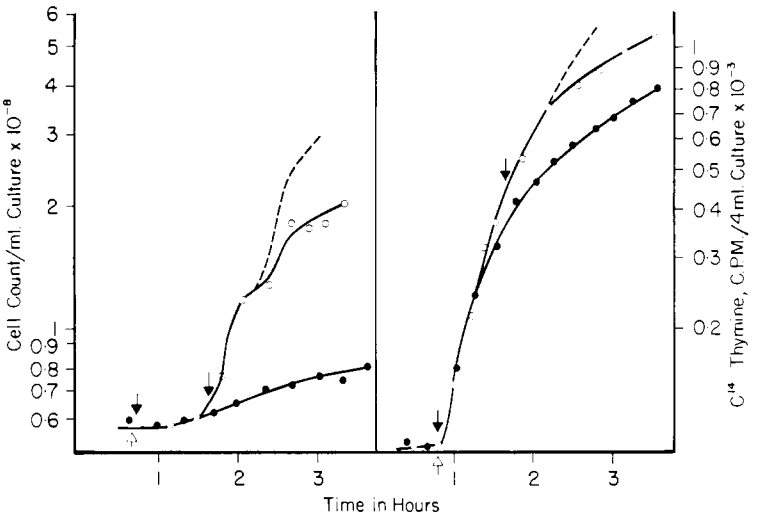

FIGURE 5: The effect of FPA (10^{-3} M) on cells counts and DNA synthesis. (↓) Time of addition of thymine. (↓) Time of addition of FPA. (●) FPA added 1 min after thymine. (○) FPA added 55 min after thymine. (---------) Uninhibited culture. The values (counts per minute) obtained prior to addition of thymine-^{14}C resulted from adding thymine-^{14}C to the cells at the time of harvesting and represent incorporation and nonspecific binding in the cells during the preparation for counting.

dry weight of cells as determined colorimetrically and by radioactivity is shown in Figure 2. The actual incorporation of uracil is presented, but identical results were obtained using guanine-^{14}C. In both cases the tendency appears to be toward a decreased ratio in the inhibited cells.

Treatment of E. coli 15$_T$- with FPA. *E. coli* 15$_T$- mutant was employed for the study of the effects of FPA on cell division. The mutant was first tested for its response to FPA and the results are shown in Figure 3. All phases of inhibited growth observed with *E. coli* B appear, including the slow exponential growth when an aliquot of a slow log culture is transferred to fresh medium containing FPA. During slow exponential growth, cell division is also maintained at the slower exponential rate. Aliquots of cultures in the linear phase were viewed under the phase-contrast microscope and the cells were found to exhibit filamentation similar to that seen with *E. coli* B. This observation is consistent with the decrease in cell count:mass ratio of inhibited cultures.

The procedure for obtaining cultures phased with respect to cell division by thymine starvation has been described in the Methods section. Although only one distinct step in cell division was observed, the system was shown to give reproducible patterns of increase in mass, RNA, DNA, and cell count. The effects of thymine starvation on the rates of increase in mass, RNA, DNA, and cell division are shown in Figures 4 and 5 as the curves obtained from uninhibited cultures. The mass of the culture and RNA increased at the exponential rate characteristic of a randomly growing culture. In some experiments a slight lag in rate of increase in mass was observed shortly after addition of thymine, as can be seen in Figure 4. This lag is increased by a longer starvation time and probably represents a decrease in viable organisms owing to thymineless death. Cultures exhibiting a lag phase longer than that shown were not studied. The cultures that were studied doubled in the number of cells over a 10-min period between 50 and 60 min after addition of thymine. After this one division the cells became random with respect to cell division within two doubling times. The cells were counted and absorbancy was determined at 12- or 15-min intervals for all

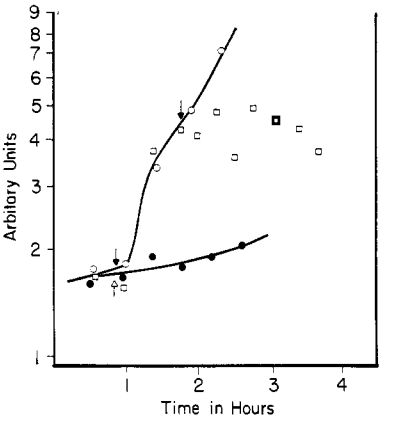

FIGURE 6: Comparison of RNA polymerase activity in inhibited and uninhibited cultures. (↑) Time of addition of thymine. (↓) Time of addition of FPA (10^{-3} M). (○) RNA polymerase activity of uninhibited phased cells. (●) RNA polymerase activity in cells with FPA added 1 min after thymine. (□) RNA polymerase activity in cells with FPA added 55 min after thymine.

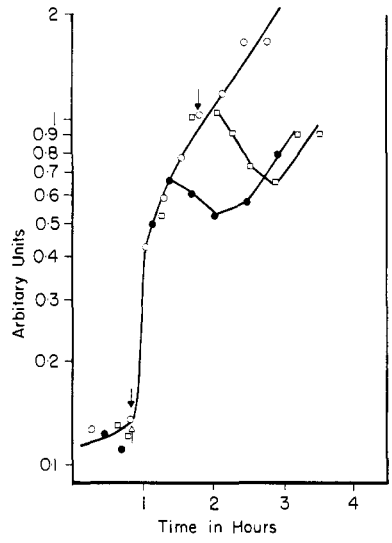

FIGURE 7: Comparison of isocitric dehydrogenase activity in inhibited and uninhibited cultures. (↑) Time of addition of thymine. (↓) Time of addition of FPA (10^{-3} M). (○) Isocitric dehydrogenase activity in uninhibited phased cells. (●) Isocitric dehydrogenase activity in cells with FPA added 1 min after thymine. (□) Isocitric dehydrogenase activity in cells with FPA added 55 min after thymine.

experiments. The rate of synthesis of DNA following thymine starvation was rapid for about 1 hr following addition of thymine. During this time the DNA doubled about four times, whereas the RNA and mass of the culture doubled once.

The effect of FPA on mass, RNA, DNA, and cell division in cultures phased for cell division is shown in Figures 4 and 5. When PFA was added 1 min after thymine addition the rate of increase in mass and RNA changed to the characteristic linear rate. Cell division was blocked and the culture proceeded directly to slow exponential growth (not shown). The rate of synthesis of DNA was rapid for several minutes before changing to that characteristic of linear growth. When FPA was added 55 min after the addition of thymine, which was at the time the cells were beginning to divide, the rates of increase in mass, RNA, and DNA changed to the linear rate. There was no inhibition of cell division cycle. Addition of FPA at 20 and 35 min after thymine gave intermediate results. There was less than a 50% increase in cell count when FPA was added 35 min after thymine.

RNA Polymerase and Isocitric Dehydrogenase in FPA-Treated Cells. In exponentially growing cultures of *E. coli* 15_T-, DNA-dependent RNA polymerase activity increases at a rate paralleling that of growth. In phased cultures RNA polymerase activity increases at a very slow rate during the period of thymine starvation. When thymine is added to the culture, the polymerase activity doubles within 35–40 min and continues to increase exponentially, as shown in Figure 6.

In contrast, when FPA is added to a culture immediately after thymine, the increase in polymerase activity is almost entirely blocked. This blocking is similar to that observed for the inhibition of cell division. Addition of the analog 55 min after thymine also results in a blocking of polymerase activity. At this time the activity of the enzyme has a little more than doubled over that present during thymine starvation. Figure 6 shows the changes in polymerase activity under these conditions. (For corresponding cell counts, see Figure 5.)

The results from studies with isocitric dehydrogenase are shown in Figure 7. When thymine was added to the starved culture, the dehydrogenase activity increased immediately and rapidly and followed the rate of DNA synthesis. The dehydrogenase activity increased about

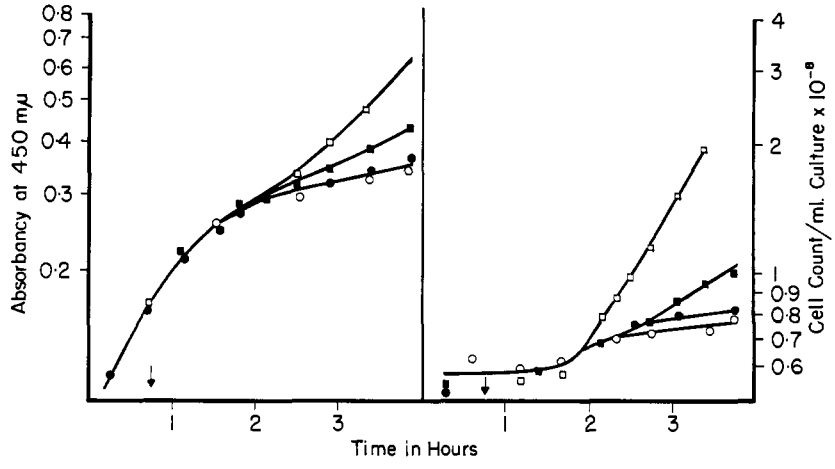

FIGURE 8: The effect of tyrosine and puromycin on FPA- (10^{-3} M) inhibited culture. (↓) Time of addition of thymine (2 μg/ml) and FPA. (■) Control to which no further additions were made. (□) L-Tyrosine (10^{-3} M) added 55 min after thymine. (○) Puromycin (5×10^{-3} M) added 55 min after thymine. (●) Tyrosine and puromycin added 55 min after thymine.

four times over the level of activity during the period of starvation before assuming an exponential rate of increase paralleling that of mass, whereas the polymerase activity only doubled. The dehydrogenase activity appeared to increase somewhat earlier than the RNA polymerase activity.

In contrast to the observations with RNA polymerase, the addition of FPA did not block the rapid immediate increase in isocitric dehydrogenase activity. After several minutes the rate of synthesis of the enzyme adjusted to the slow exponential growth rate. When FPA was added 55 min after the addition of thymine, the dehydrogenase activity did not exhibit an immediate increase but decreased for 1 hr and then was synthesized at the slow exponential rate. When FPA was added to a randomly growing culture the formation isocitric dehydrogenase was blocked and the progress curve for activity was like that obtained when FPA was added 55 min after thymine.

Effect of the Presence of Puromycin on the Ability of Tyrosine to Reverse FPA Inhibition. The addition of tyrosine to a culture growing in the presence of FPA results in a return of the culture to a nearly normal exponential rate. Similar results were obtained by the addition of phenylalanine or tyrosine to a phased culture that had been treated with FPA. There was an immediate increase in cell count when the natural amino acid was added. As shown in Figure 8 the effect of tyrosine is blocked by puromycin. This result suggests that the reversal of the inhibition by tyrosine requires protein synthesis.

Discussion

The characteristic growth phases of the strains of *E. coli* studied here in the presence of FPA are the initial linear phase and the subsequent slow log phase. The rate of increase in mass and in general the rates of DNA and RNA synthesis follow this sequence. Inhibition by FPA of phased cells also results in the appearance of these phases, although the transition from normal to slow log growth seems to be more direct when cells are inhibited at the end or beginning of the cell cycle (*i.e.*, 55 min after addition of thymine). Whatever form of adjustment is made in these cells to permit growth to continue exponentially, therefore, would appear to take place during the early part of the cell cycle.

The results of the addition of FPA to phased cultures at various times after thymine addition suggest a time-related sensitivity of the organism to the presence of the analog. This is evidenced in the rate changes of cell division. Here, the presence of the analog fails to interfere with the process when the cells are sufficiently close to the point of division.

Although it has been shown that FPA causes both inhibition and repression of 3-deoxy-D-*arabino*-heptulosonic acid 7-phosphate synthetase (Smith *et al.*, 1964; Previc and Binkley, 1964b), an early enzyme in the aromatic biosynthetic pathway, it seems probable that there is some other point of sensitivity to the analog in view of the immediate and extensive blocking of RNA polymerase activity as well as cell division in the transition period. In contrast the activity of the respiratory enzyme isocitric dehydrogenase in phased cells is not blocked immediately, nor is the synthesis of DNA immediately affected. A separation between the events of DNA synthesis and cell division is thus accomplished. This effect probably results from alterations owing to thymine starvation.

Results of the experiments in which FPA inhibition was reversed indicate that protein synthesis is involved. Reversal of inhibition of cell division by the addition of phenylalanine or tyrosine is accomplished quite rapidly. The conclusion that synthesis of one or more proteins occurs prior to the increased rate of cell division when the natural amino acid is added is based on the results obtained when puromycin was added at the same time as the amino acid. No increase in cell division was observed under these conditions where it would be expected that at least some increase in cell count would occur if protein synthesis were not involved.

Studies on thymine starvation (Pritchard and Lark, 1964) have shown premature initiation of chromosome replication. When thymine was added back to the culture, DNA synthesis proceeded from both the origin and the place where synthesis had ceased during starvation. A model relating to this phenomenon (Jacob *et al.*, 1963) (Lark and Lark, 1964) is one in which two proteins, one structural and the other termed an initiator, are involved in the mechanism. Later (Lark and Lark, 1965) it was suggested that the regulatory mechanism may be coupled in some way to the cell division process. Recently a more detailed model involving the initiator proteins and structural proteins has been presented (Lark, 1966; Lark and Lark, 1966).

Our results can be explained by assuming that FPA is interfering with initiation of replication but allows the completion of a chromosome replication cycle. This could result from the production of a defective initiator protein or structural protein. In studies to be reported elsewhere it has been found that cells in the process of adjusting accumulate UDPAG and that the cell wall membrane fraction of cells in the linear phase is deficient in *N*-acetylglucosamine. Cells in the slow log phase possess a normal amount of the amino sugar in this fraction. There is the possibility that FPA is causing a structural defect in the glycoproteins and thereby preventing attachment of the DNA to the structural site.

The inhibition of RNA polymerase acticity by the analog suggests that this enzyme is involved in the initiation process or is dependent on it. The model suggested (Lark, 1966) to explain the effect of amino acid starvation and phenylethanol inhibition can also be used to explain the inhibition by FPA. It is interesting that FPA and phenylethanol give identical growth kinetics in random cultures of *E. coli* B, *i.e.*, a linear phase followed by a slow exponential phase.

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Characterization of the Ribonucleic Acid Synthesized in an Isolated Nuclear System from Rat Heart Muscle*

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ABSTRACT: Ribonucleic acid (RNA) synthesized by a purified nuclear preparation from rat heart muscle was examined by sucrose density gradient analysis and by nearest neighbor frequency studies of the bases. Examination of the RNA sedimentation profile after a short incubation period revealed some high molecular weight material. This was particularly so in the presence of a high concentration of ammonium sulfate. Nearest neighbor analyses showed that the species of RNA synthesized under conditions of low ionic strength had a base composition similar to ribosomal ribonucleic acid (rRNA) or its precursor. With ammonium sulfate at high ionic strength (0.6 M) the predominant species was deoxyribonucleic acid-like RNA.

Whereas the importance of DNA-dependent RNA polymerase (EC 2.7.7.6) is fairly well established in the genetic control of protein synthesis, and its properties have been extensively studied in bacterial and mammalian systems (Weiss, 1960; Hurwitz *et al.*, 1962; Chamberlin and Berg, 1962; Nakamoto *et al.*, 1964; Tsukada and Lieberman, 1964; Widnell and Tata, 1964a,b), the nature of the RNA synthesized by mammalian nuclear preparations has not yet been clearly defined.

Stimulation of mammalian nuclear RNA polymerase activity upon the addition of various salts, notably ammonium sulfate, to the assay medium was first described by Goldberg (1961). Recently it has been stressed by Breuer and Florini (1966) that the stimulatory effect of $(NH_4)_2SO_4$ may be due to increased activity of the primer component of the nuclear system. A similar conclusion was reached by Pogo *et al.* (1966), from their experiments involving regenerating liver cell nuclei. So far, it has been assumed on the basis of indirect evidence that the RNA produced under standard reaction conditions involving Mg^{2+} ions and low ionic strength is like rRNA and that in the presence of ammonium sulfate (with high ionic strength) the product more closely resembles complementary RNA. Furthermore, both Tata and Widnell (1966) and Liao *et al.* (1966) have suggested that differential synthesis

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