EFFECT OF ANTIBIOTICS AND ANTIMETABOLITES ON THE INDUCTION OF L-ARABINOSE ISOMERASE IN *SALMONELLA TYPHIMURIUM*

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ABSTRACT

The effects of three antibiotics (mitomycin C, actinomycin D and rifampin) and the antimetabolite (fluorouracil) on the induction of L-arabinose isomerase in *Salmonella typhimurium* have been studied. Fluorouracil, at a concentration of 10 μg/ml which does not interfere with growth of *S. typhimurium* during the period of study, interferes with the induction of the enzyme which may be due to the synthesis of faulty *m*RNA. Under the same conditions fluorouracil has no effect on the overall rate of protein synthesis. Mitomycin C (5 μg/ml) has no effect on the induction of the isomerase. As expected, actinomycin D and rifampin interfere with the induced synthesis of L-arabinose isomerase. The results indicate that the induction of L-arabinose isomerase is controlled at the level of transcription.

INTRODUCTION

THE studies on the induction and repression of enzymes in bacteria have helped to a great extent to understand the control mechanism at the genetic level. Most of this work has been carried out with the *lac* operon in *Escherichia coli*. These studies led to enunciation of the well-known concept of Jacob and Monod[1-2] which turned out to be more or less correct in great details. Considerable genetic investigations have been carried out with the *ara* operon in *E. coli*[3-4]. Catabolite repression of the synthesis of L-arabinose isomerase in *Salmonella typhimurium* and its reversal by cyclic 3′, 5′-AMP have already been reported from this laboratory[5]. We have also studied the entry of L-arabinose in *S. typhimurium*[6].

The antibiotics and antimetabolites which block some specific steps of protein and RNA synthesis have been widely used to understand the mechanism of genetic control of protein synthesis. It was therefore of interest to study the effects of these antibiotics and antimetabolites on the induction of L-arabinose isomerase. In the present investigation, fluorouracil, actinomycin D, mitomycin C and rifampin have been used to show that the *ara* enzyme synthesis is regulated at the level of transcription.

MATERIALS AND METHODS

Bacterial Strain.—*S. typhimurium* LT2 was kindly supplied by Prof. Myron Levine of the University of Michigan, Ann Arbor, Michigan, U.S.A.

Chemicals.—5-Fluorouracil was a gift from Prof. S. P. Champe of the Institute of Microbiology, Rutgers State University, Brunswick, New Jersey, U.S.A. Mitomycin C was kindly supplied by Prof. Y. Takagi of the Kyushu University, Fukuoka, Japan. Rifampin was a gift from Dr. J. Gelzer, Director, Microbiology Division, CIBA Pharmaceutical Company, New Jersey, U.S.A. Actinomycin D was obtained from Merck, Sharp and Dohme Research Laboratories, New Jersey, U.S.A. L-Arabinose was obtained from Calbiochem, Los Angeles, U.S.A. All other chemicals were commercial preparations and were of analytical grade.

Growth medium, Growth of S. typhimurium, *Induction of L-arabinose isomerase, Assay of L-arabinose isomerase and EDTA treatment of cells for induction experiments* have been described elsewhere[5].

Measurement of the overall rate of protein synthesis.—The overall rate of protein synthesis was measured by following the incorporation of C^{14}-L-phenylalanine into *S. typhimurium*. Cells were grown from an overnight grown inoculum as described before[5]. When the cell suspension reached an absorbancy of 0·12 ($1·6 \times 10^8$ cells/ml), 16 μmoles of L-arabinose and 0·05 μmole of C^{14}-L-phenylalanine (having 5×10^4 counts/min) were added per ml of the cell suspension. At 30th minute following the addition of C^{14}-L-phenylalanine, fluorouracil was added at a final concentration of 10 μg/ml. At different time intervals, aliquots (1 ml) were collected in pre-chilled tubes containing 1 ml of 10% trichloroacetic acid. The mixtures were kept in ice for 30 min and the trichloroacetic acid insoluble precipitates were collected by filtration through millipore filters. Each filter was washed with 25 ml of cold 5% trichloroacetic acid, mounted on planchet, dried and counted in a windowless gas flow counter of Bhabha Atomic Research Centre, Trombay, India.

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Results

Effect of fluorouracil on the induction.—Fluorouracil is known to be incorporated into RNA in place of uracil and thus to produce defective mRNA[7,8]. This pyrimidine analogue at a concentration of 10 μg/ml had little effect on the growth of *S. typhimurium* up to about 45 min following its addition; at which time growth is inhibited, and finally the growth completely halts after about 75 min (results not presented). Fluorouracil (10 μg/ml) was added 30 min following the addition of the inducer and its effect on the enzyme induction was studied for another 40 min (Fig. 1). The induction of L-arabinose isomerase was inhibited immediately after the addition of fluorouracil.

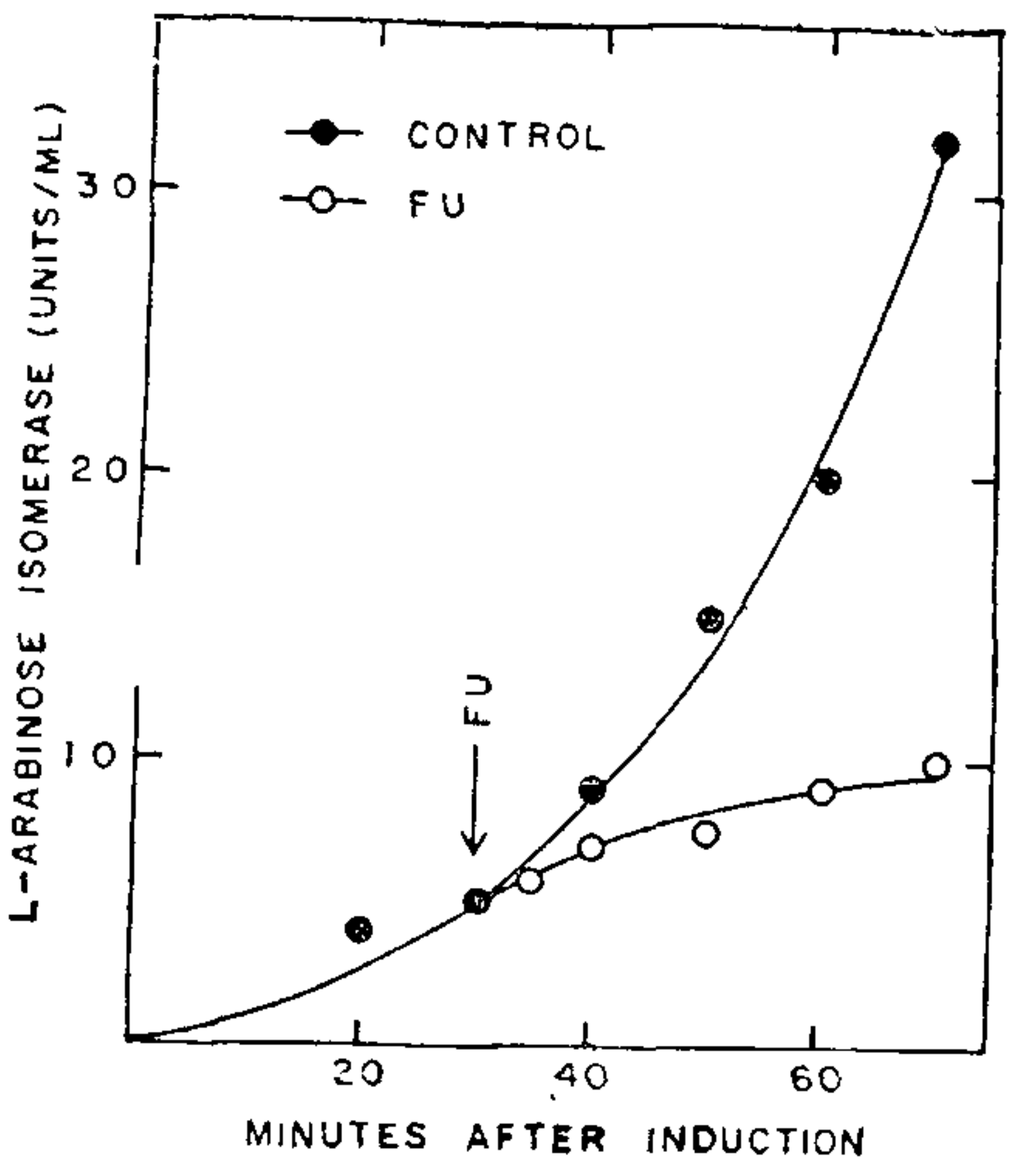

FIG. 1. *Effect of fluorouracil on the induction of L-arabinose isomerase.* Samples were removed at the indicated time intervals for the assay of the enzyme. Fluorouracil (10 gμ/ml) was added at 30th minute following the addition of the inducer.

As discussed already, fluorouracil is incorporated in place of uracil and thus produces defective mRNA. This may lead to cessation of protein synthesis or the protein synthesised from the faulty mRNA may not be enzymatically active. In the first case the overall rate of protein synthesis will be affected in presence of fluorouracil whereas in the second case fluorouracil will have no effect. It is clear from results presented in Fig. 2 that fluorouracil had little effect on the overall rate of protein synthesis at least for 40 min, the period for which its effect on the induction of L-arabinose isomerase was studied.

Effect of mitomycin C on the induction of L-arabinose isomerase.—Mitomycin C is known to interfere with the duplication of DNA but does not normally inhibit RNA and protein synthesis[9]. As expected, the antibiotic (5 μg/ml) had no effect on the induction process. The level of the enzyme increased up to 30 min but after that there was no increase of enzyme level (Fig. 3). The action of the antibiotic was in parallel with its effect on the growth of *S. typhimurium*. In presence of 5 μg/ml of mitomycin C the growth continued at the normal rate for about 30 min and then completely ceased (results not presented).

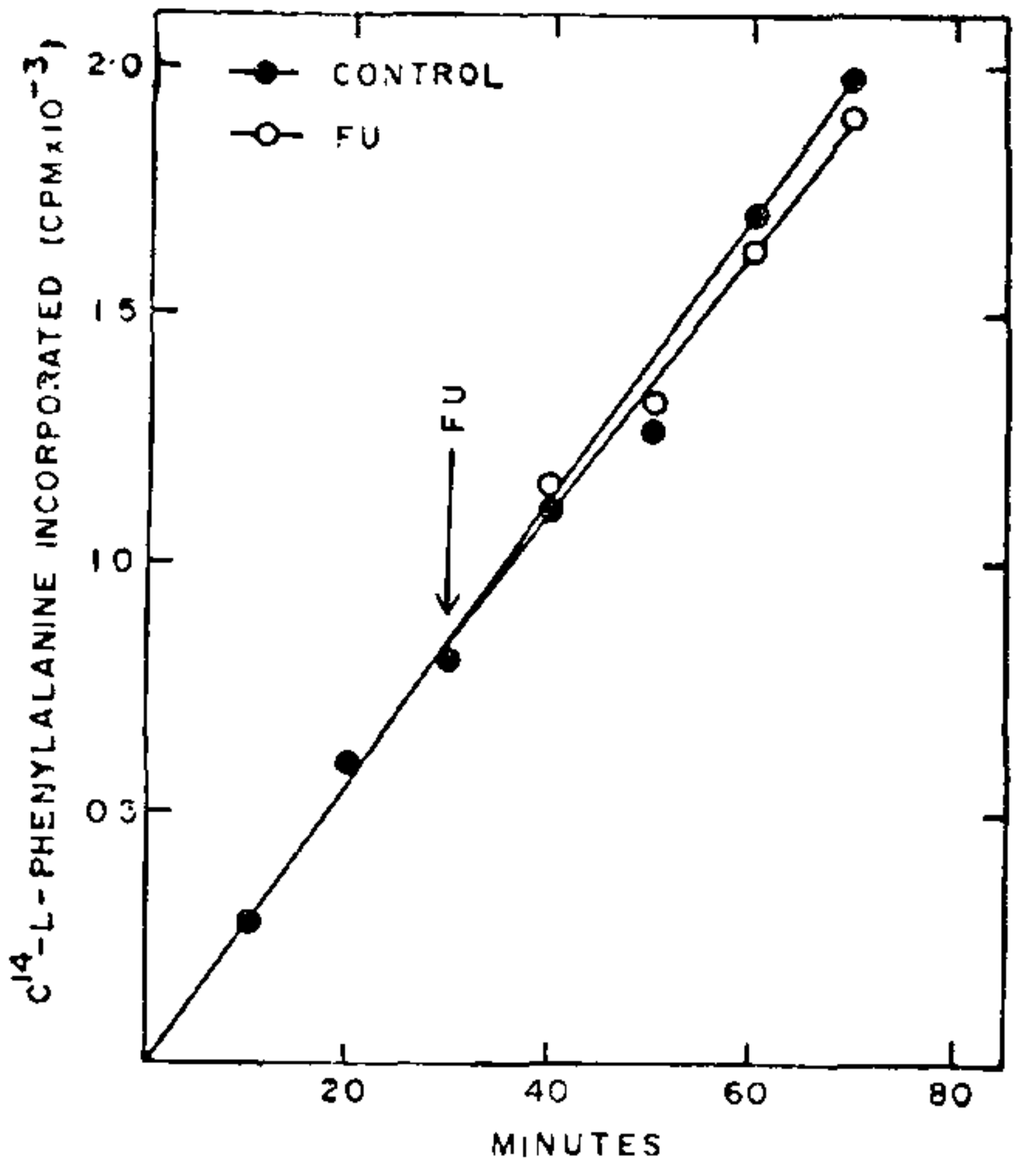

FIG. 2. *Effect of fluorouracil on the overall rate of protein synthesis.* The rate of protein synthesis was measured as described under 'Materials and Methods'. Flucrouracil (10 μg/ml) was added at 30th minute following the addition of L-arabinose and C^{14}-L-phenylalanine.

The lack of interference by mitomycin C on enzyme induction for 30 min may be due to the weak permeability of the compound in *S. typhimurium*. This was tested by studying the effect of the antibiotic on the induction of L-arabinose isomerase in EDTA-treated cells. Results presented in Fig. 4 clearly indicate that in EDTA-treated cells a longer period (60 min; compare Figs. 3 and 4 *a*) is required for mitomycin C to exert its effect on the induction of the isomerase. The kinetics of enzyme induction in presence of mitomycin C run parallel with that of the growth rate (Figs. 4 *a* and *b*). The growth rate in cells treated with EDTA is slower than that of non-treated cells. Therefore, the delay in stopping the induced syn-

thesis of L-arabinose isomerase is not due to the permeability of *S. typhimurium* cells to mitomycin C.

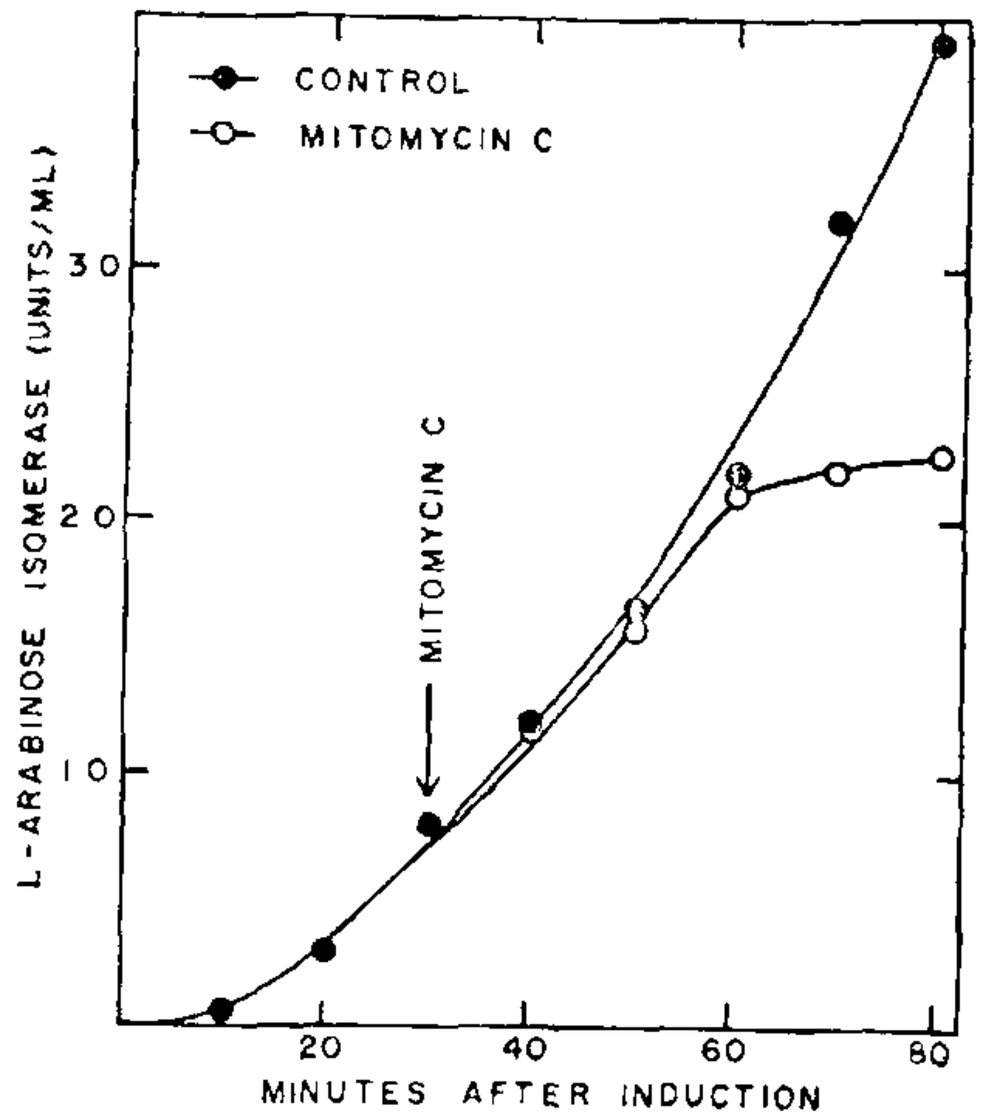

FIG. 3. *Effect of mitomycin C on the induction of L-arabinose isomerase.* Mitomycin C (5 μg/ml) was added at 30th minute following the addition of the inducer.

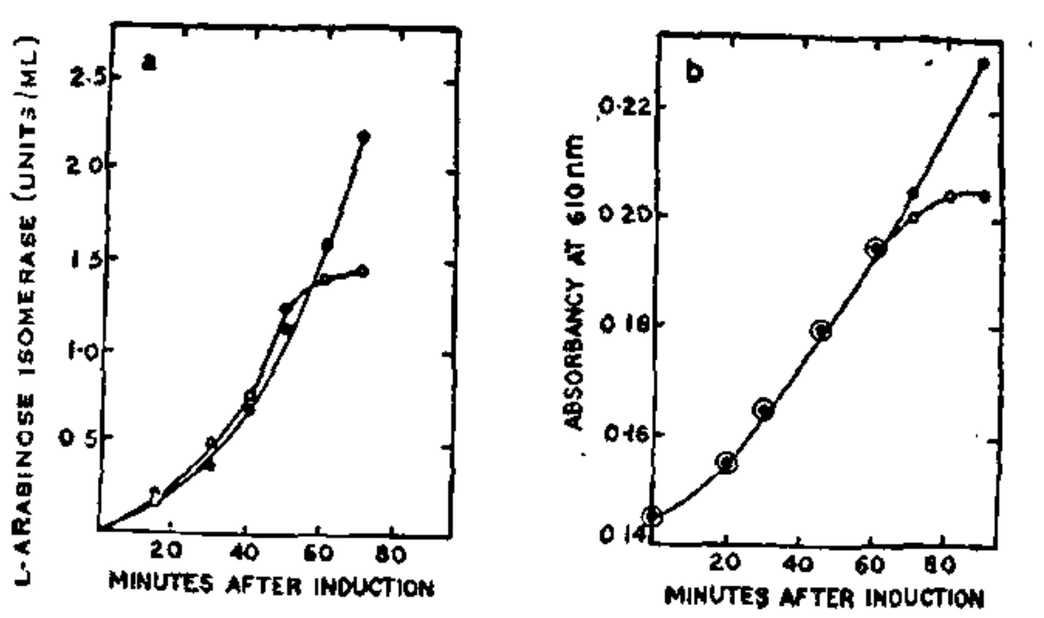

FIG. 4. *Effect of mitomycin C on the induction of L-arabinose isomerase* (a) *and growth of S. typhimurium* (b) *in .EDTA-treated cells.* The EDTA-treatment of the cells was done as described earlier[5]. Mitomycin C (5 μg/ml) was added along with the inducer. -●-, control; -O-, mitomycin C.

Effect of actinomycin D on the induction.—Actinomycin D is a potent inhibitor of DNA-dependent RNA synthesis[10-13]. Being a Gram-negative organism *S. typhimurium*, however, was insensitive to the action of actinomycin D. The cells could, however, be made sensitive to the action of actinomycin D by treatment with EDTA[14]. The effect of actinomycin D (20 μg/ml) on the induction of L-arabinose isomerase was studied for 70 min both in untreated and EDTA-treated cells. The results are presented in Table I. As expected, actinomycin D had no effect on the induction in untreated *S. typhimurium*. The induction in EDTA-treated cells was, however, inhibited to an extent of 60% by actinomycin D.

TABLE I

Effect of actinomycin D on the induction of L-arabinose isomerase in normal and EDTA-treated cells

Cells	Actinomycin D	
	−	+
	(Units/ml)	
Untreated	3·1	3·0
EDTA-treated	2·3	0·9

Actinomycin D (20 μg/ml) was added along with the inducer and the induction was carried out for 70 min.

Effect of rifampin on the induction.—Rifampin is a derivative of rifamycin which is known to interfere with the RNA synthesis by binding directly with DNA-dependent RNA polymerase[15,16]. Therefore it was expected that this antibiotic would interfere with the enzyme induction. The effect of varying concentration of rifampin on the induction was studied first (Fig. 5). At a concentration of 20 μg/ml of rifampin, the enzyme production was inhibited to an extent of 83%. A comparatively high concentration of rifampin (50 μg/ml) was chosen to study the time course of its action (Fig. 6). Rifampin completely blocked the enzyme induction immediately following its addition.

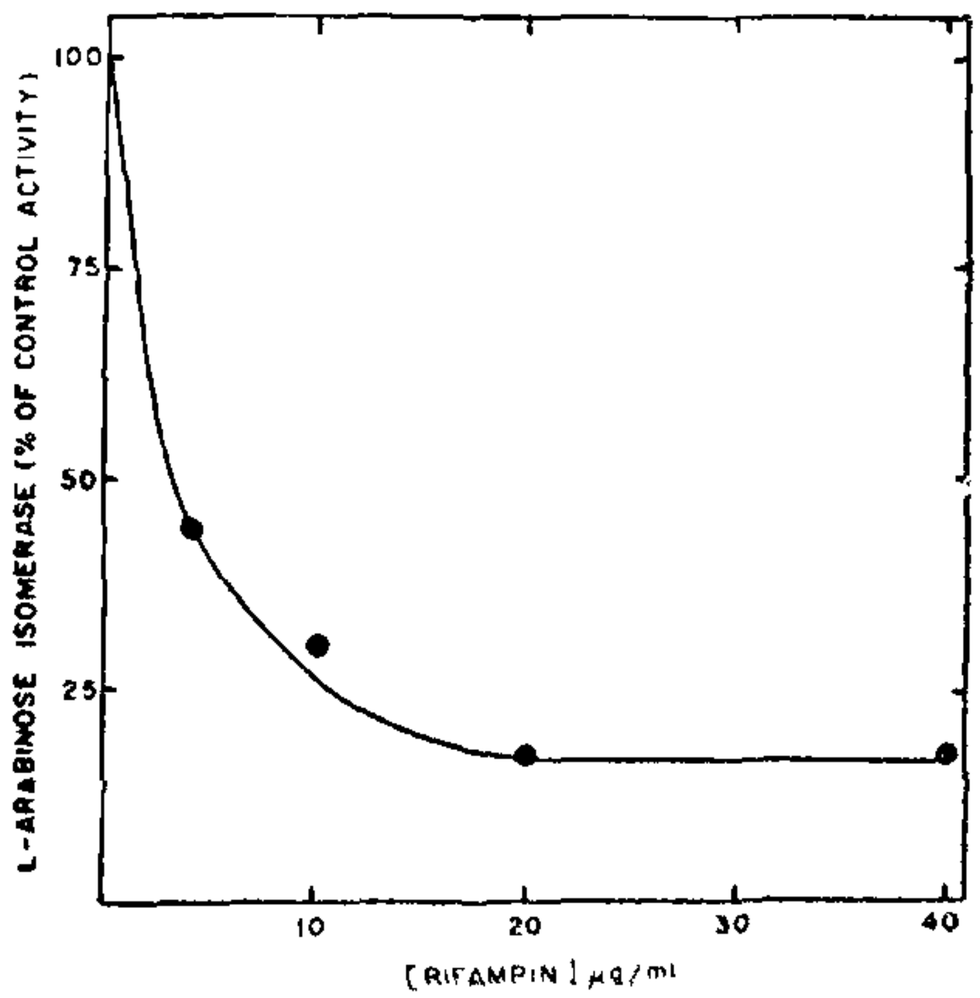

FIG. 5. *Effect of varying concentration of rifampin on the induction of L-arabinose isomerase.* Induction was carried out for 70 min. Varying concentrations of rifampin as indicated was included along with the inducer in the medium. Control activity (100 per cent) denotes the amount of enzyme induced in the absence of rifampin.

Discussion

L-Arabinose induces the enzyme L-arabinose isomerase in *S. typhimurium* growing in MM having glycerol as the carbon source. The basic features of the process of induction of L-arabinose isomerase are the same as that of the induction of β-galactosidase[5]. However, in certain aspects, specially with reference to both positive and negative control exerted by the inducer, the L-arabinose system seems to be somewhat different from β-galactosidase system[3,4]. Though it seems from the recombination studies that the control involves the regulation of synthesis of *ara* mRNA[17], no direct evidence for this has yet been found. An attempt has been made in the present investigation to find out the level at which this control is exerted in the *ara* operon.

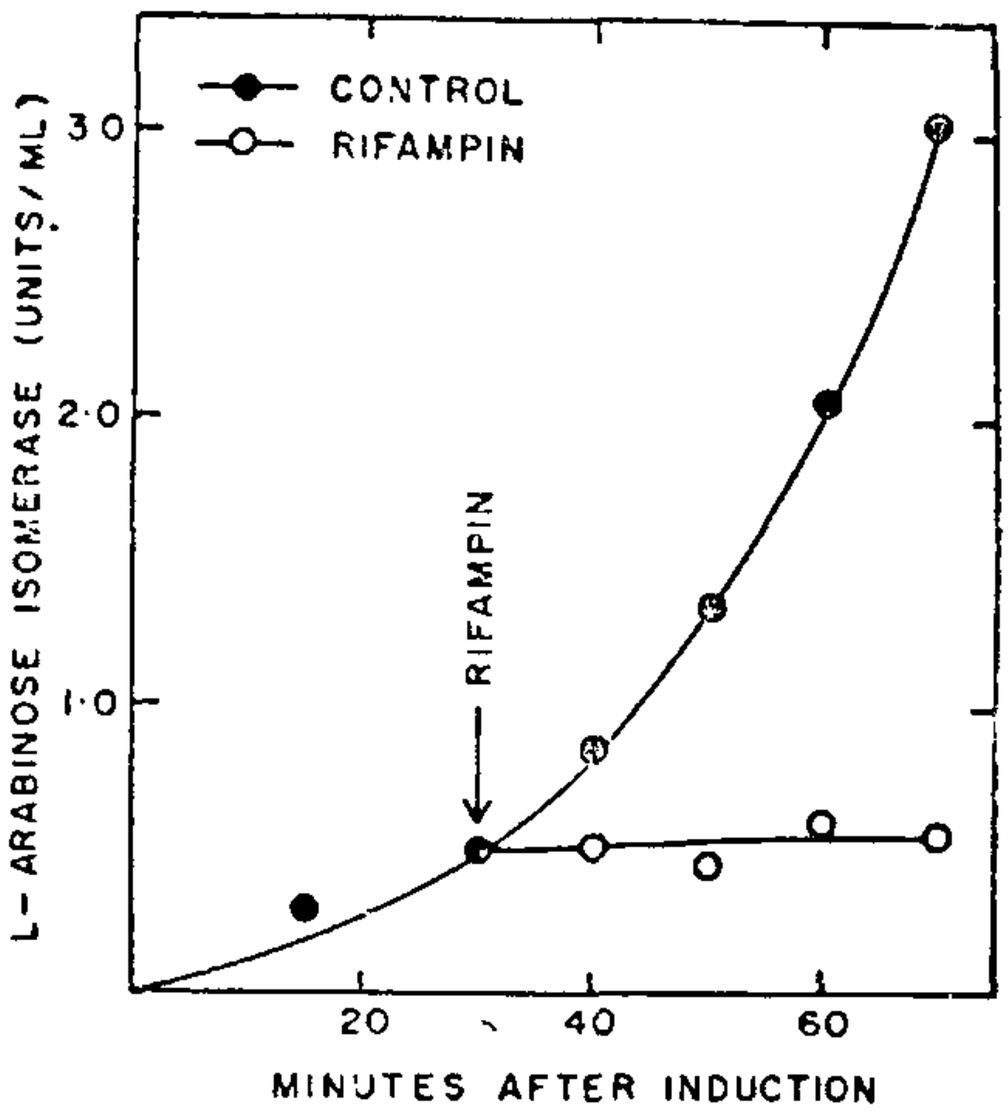

FIG. 6. *Effect of rifampin on the kinetics of induction of L-arabinose isomerase.* Rifampin (50 μg/ml) was added at 30th minute following the addition of the inducer.

The induction of the enzyme is stopped in presence of the antimetabolite fluorouracil (Fig. 1). Even at a concentration (10 μg/ml) which does not interfere with the rate of growth of the microorganism during the period of study (results not presented), very little active enzyme is synthesised. The failure to produce active enzyme in presence of fluorouracil is most probably not due to lack of transcription or translation, but is the result of faulty translation because the rate of overall protein synthesis as measured by C^{14}-L-phenylalanine incorporation is not affected in presence of fluorouracil (Fig. 2). The effect of fluorouracil on the synthesis of L-arabinose isomerase in *S. typhimurium* resembles its effect on β-galactosidase synthesis[18-20]. However, it differs from that on L-arabinose isomerase synthesis in *L. plantarum* as reported earlier from this laboratory[21]. In *L. plantarum* fluorouracil has no effect on L-arabinose isomerase synthesis.

Mitomycin C (5 μg/ml) which interferes with the duplication of DNA does not interfere with the induction of the enzyme up to 30 min (Fig. 3) following its addition. After 30 min the growth of the microorganism is stopped (results not presented), hence there is no further synthesis of the enzyme. Even when *S. typhimurium* cells were treated with EDTA to increase their permeability to mitomycin C, the effect of the antibiotic on the induction of L-arabinose isomerase is in parallel with its effect on the growth of the microorganism (Fig. 4). The nongrowing condition of the cells is responsible for no further synthesis of the enzyme. The results obtained with *S. typhimurium* are in conformity with those reported in case of the same enzyme from *P. pentosaceus* and *L. plantarum*[21,22]. It should be mentioned here that penicillinase induction in *S. aureus*[23] and β-galactosidase induction in *E. coli*[24,25] cannot take place if the cells are treated with mitomycin C.

The antibiotic actinomycin D is widely used to study the direct involvement of mRNA. Being a Gram-negative organism *S. typhimurium* is not sensitive to actinomycin D. However, in EDTA-treated cells the induction of enzyme is inhibited in presence of actinomycin D (Table I). The slight difference in the levels of the enzyme in untreated and treated cells (in absence of actinomycin D) is a reflection of the difference in the growth rate in the two cases.

As expected, rifampin completely blocks the enzyme induction (Fig. 6). The induction of the enzyme decreases exponentially with the increase in the concentration of rifampin (Fig. 5). In presence of 20 μg/ml of the antibiotic, the level of L-arabinose isomerase is only 17% of that in absence of antibiotic. With further increase in the concentration there is no more decrease in the level of the induced enzyme.

The results presented in this paper clearly show that as in case of most inducible enzymes, L-arabinose isomerase induction also is regulated at the level of transcription.

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ESTABLISHMENT OF SYMBIOSIS *IN VITRO*, BETWEEN *RHIZOBIUM* AND PEA (*PISUM SATIVUM*) ROOT CALLUS

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ABSTRACT

Pea (*Pisum sativum*) root callus was inoculated with *Rhizobium leguminosarum* and studied for the establishment of symbiosis. Infection thread-like structures were observed penetrating the callus intercellularly and bacteria and bacteroid-like bodies were seen in the cells of the callus tissue. Nitrogenase activity was detected in some samples of calli indicating the nitrogen fixing ability of the infected callus tissue.

INTRODUCTION

THE formation of infection threads and nodules in legume-*Rhizobium* association has been studied in whole plants[1] and in isolated roots[2] under aseptic conditions. An unsuccessful attempt to establish symbiosis in isolated plant tissue (callus) was made by Veliky and La Rue[3] in 1967. This was followed by a successful attempt by Holsten *et al.*[4] in 1971, using soybean root callus infected with *R. japonicum*. Nitrogenase activity was detected in infected calli (by acetylene reduction technique) and the tissue contained infection thread-like structures and bacteroid-like cells. The results reported in this paper relate to our work with pea root callus infected with *R. leguminosarum*, using the method followed by Holsten *et al.*[4] with slight modifications.

MATERIALS AND METHODS

Seeds of *Pisum sativum* var. *baunville* were surface sterilised with cetavlon (1%) and germinated on Murashige-Skoog's[5] (MS) basal medium. The roots were cut off when 2–3 cm long and transferred to MS medium supplemented with 2, 4 - D and kinetin. Callus formation was observed after 1 month. The callus was transferred to MS basal medium and allowed to remain there for 5 days. Pure cultures of *R. leguminosarum* were isolated from pea root nodules by conventional procedure[6] and maintained on yeast extract mannitol agar (YEMA) slants.

One ml of a YEM broth culture containing actively growing bacteria was transferred to each test-tube containing MS basal liquid medium with actively growing callus and incubated in darkness at 25 ± 2° C. Uninoculated calli served as controls. After seven days incubation, the callus mass was washed twice in MS basal liquid medium under aseptic conditions. The solution containing the callus mass was filtered through sterile cheese cloth and calli transferred from the cheese cloth to MS basal solid medium. They were incubated for