EFFECT OF ENERGY SUPPLY ON ENZYME INDUCTION BY PYRIMIDINE REQUIRING MUTANTS OF ESCHERICHIA COLI

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A study of the formation of induced (adaptive) enzymes in purine or pyrimidine requiring mutants of *Escherichia coli* was reported in a previous publication (Pardee, 1954). The idea that synthesis of ribonucleic acid (RNA) is essential for protein synthesis is supported by the inability of the bacterial mutants to form protein and several enzymes, including β-galactosidase, in the absence of purines or pyrimidines. All of these experiments were performed in a medium which contained an energy supply other than the inducer. Early in the work, it was noted, however, that in a medium which lacked both an energy supply and the nucleic acid component, formation of the induced enzyme did proceed to a considerable extent. The explanation of this phenomenon seems to lie in certain complications not pertinent to the relation of nucleic acid and protein synthesis and is the subject of this communication.

METHODS

E. coli, mutant 9661-01 (purineless), was isolated by Dr. R. Guthrie. *E. coli*, strain ML, was provided by Dr. G. S. Stent. Other strains and experimental methods were described in the previous paper (Pardee, 1954).

"Minimal salts" contained per liter: 7 g K_2HPO_4, 2 g KH_2PO_4, 0.5 g Na_3 citrate·$5H_2O$, 0.1 g $MgSO_4 \cdot 7H_2O$, and 1 g $(NH_4)_2SO_4$. A carbon source, usually at 0.5 per cent concentration, was added to make "minimal medium".

Nucleic acids were separated, and the nucleotides were chromatographed for P^{32} determination by the method of Magasanik *et al.* (1950). The purines and pyrimidine nucleotides were isolated and estimated by the method of Smith and Markham (1950), and the free bases by the method of Marshak and Vogel (1951).

The Warburg apparatus was used to measure O_2 uptake in the conventional manner. Measurements of radioactivity of P^{32} were made on dry samples by means of an end window Geiger-Müller counting tube, and C^{14} was determined with a gas-flow counter.

Cultures were frequently tested on minimal agar plates to be certain that mutants had not reverted.

RESULTS

Examples of enzyme induction by mutants in the absence of an energy source. In figure 1 the rates of oxygen uptake by mutant 9661-01 in various media are presented. These data show a marked increase in ability to oxidize lactose, but not glucose, in the absence of guanine. This experiment has been repeated with a variety of purine or pyrimidine requiring mutants, or with *E. coli*, strain B, in a medium lacking phosphate. The cells were originally grown on a variety of carbon sources and then induced to oxidize glycerol, lactose, lactate, or melibiose; and the same type of result has always been obtained. In the absence of the necessary growth factor a limited ability to oxidize the new carbon source appeared, and there was no increase in ability to utilize the compound on which the cells were grown.

The above results were obtained with intact cells. Since it is known that maximal enzyme activities often differ from the activities of intact cells, it seemed desirable to study the induced enzyme content of broken cells. Assays were performed on toluene treated cells with *o*-nitrophenyl-β-D-galactoside (NPG) as the substrate using the modification of Koppel *et al.* (1953). Results are shown in figure 2 for cells of mutant 6386 (pyrimidineless) induced by melibiose to form β-galactosidase at several concentrations of uracil. In the absence of glycerol, uracil is apparently not required for induction. By contrast, induction proceeded in the presence of glycerol only until added uracil was exhausted, as found previously (Pardee, 1954). In the absence of uracil, total protein per ml increased only about 5 per cent

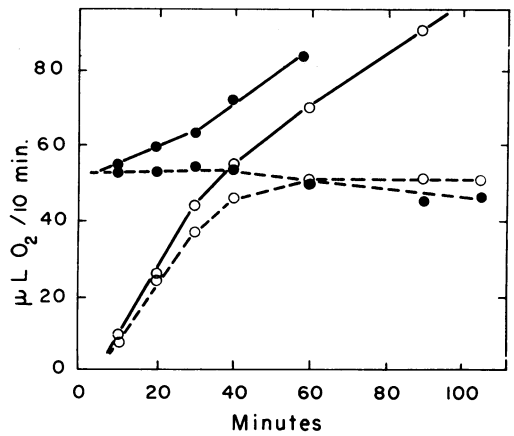

Figure 1. Oxidation of glucose (●) or lactose (○) by a purine requiring strain of *Escherichia coli* previously grown on glucose (——— plus purine; — — — minus purine).

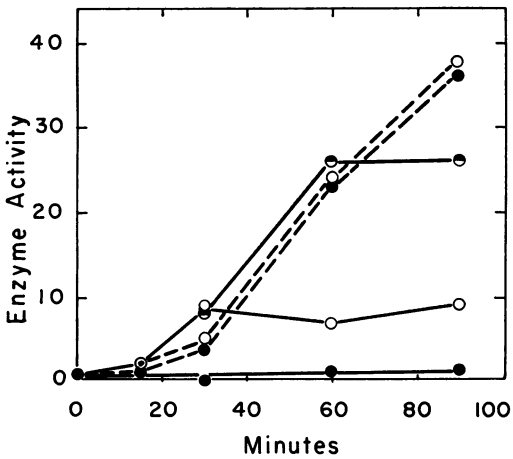

Figure 2. Induction of β-galactosidase by melibiose by a pyrimidine requiring mutant of *Escherichia coli* previously grown on glycerol (——— glycerol present; — — — glycerol absent; ●, no uracil; ○, 0.6 μg/ml uracil; ◓, 1.2 μg/ml uracil).

in 60 min as compared to an increase of 100 per cent in the presence of uracil. Purine requiring mutants in the absence of purines and *E. coli*, strain B, in a phosphate deficient medium similarly showed induction, as measured by NPG, in the absence of an external energy supply. Either lactose or melibiose could serve as inducer.

Induction in the absence of an energy supply did not occur in all cases. With *E. coli*, strain ML, which cannot grow on melibiose, induction of β-galactosidase synthesis by melibiose was observed only in the presence of an external energy source. Similarly, in *E. coli*, strain B, the *cis*- or *trans*-enol β-D-galactosides of methylacetoacetate are inducers for β-galactosidase only in the presence of an added energy supply. The *cis* compound is hydrolyzed by *E. coli*, but the *trans* is an inhibitor of hydrolysis (Ballou *et al.*, 1952).

An almost complete separation of enzyme induction and growth was obtained. This can be seen if one plots the increase of enzyme activity against the increase of protein (approximately, increase of cell material), according to Monod *et al.* (1952). The slope represents the rate of enzyme synthesis relative to growth processes (figure 3). A very much steeper slope (approximately 10 times) for induction by melibiose was obtained in the absence of glycerol and uracil than in their presence, showing that induction occurred in preference to growth under the former conditions as compared to the latter. This was also true in the presence of uracil. This result is not a property peculiar to the mutant as it was also observed with *E. coli*, strain B.

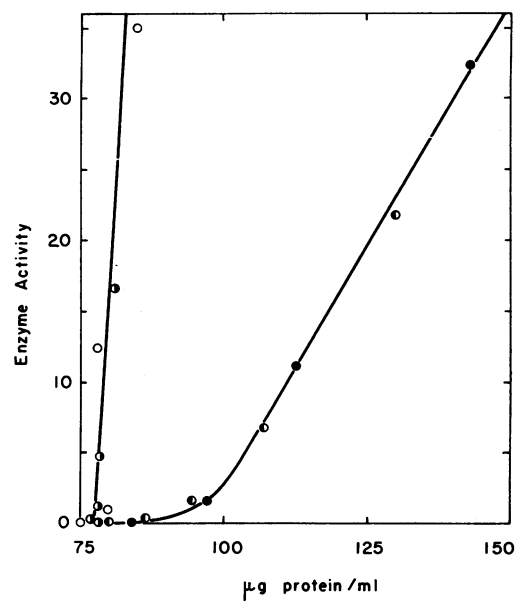

Figure 3. β-Galactosidase plotted against protein content at various times during induction by melibiose. ○ Strain 6386 on minimal salts; ● strain 6386 on minimal medium plus uracil; ◑ *E. coli*, strain B, on minimal salts; ◐ *E. coli*, strain B, on minimal salts plus glycerol.

Two separate observations are contained in these results. The first is the almost complete separation of induced enzyme synthesis and growth (figure 3). A satisfactory explanation of this awaits further study. The purpose of the present paper is to explain the second observation: Why the mutants can form induced enzymes in a deficient medium in the absence of an added energy source but not in its presence. The work to be described was largely performed with pyrimidine requiring mutants.

The influence of energy supply on enzyme induction. The rapid utilization of traces of uracil for general growth processes can explain the inability of mutants to form induced enzymes in the presence of an external energy supply and in the absence of purines or pyrimidines. It was found that an added energy source causes added uracil to disappear from the medium at least five times as rapidly as in minimal salts.

Remarkably low concentrations of an external energy source can prevent induction. For example, 50 μg/ml of glycerol prevented induction to 2 mg/ml of melibiose (in the absence of uracil, by glycerol grown mutant 6386), and even 17 μg/ml of glycerol inhibited induction for about 10 minutes (figure 4). Since the duration of inhibition was approximately the time required for removal of the glycerol by oxidation (as calculated from measurements on the uptake of oxygen), it seems that very low levels of glycerol (5×10^{-4} M or less) are able to inhibit induction completely. By comparison 10 times this concentration of arsenate or azide was required.

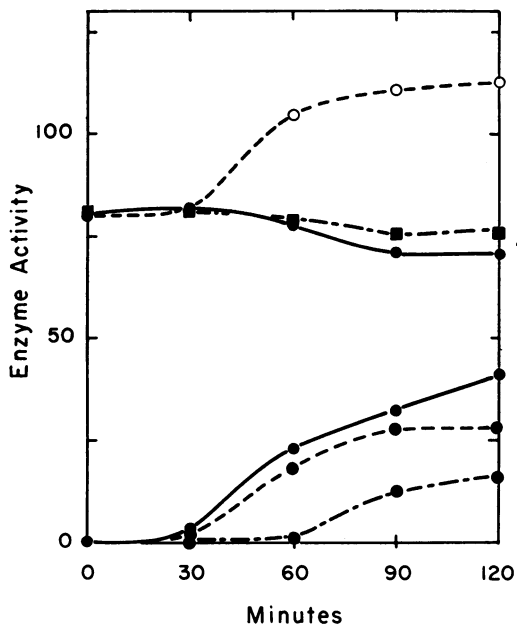

Figure 4. β-Galactosidase formation in the absence of pyrimidines by a pyrimidine requiring mutant of *Escherichia coli*. Top 3 curves obtained with cells previously grown on glycerol plus lactose (○ induction by melibiose; ■ induction by melibiose plus lactose; ● induction by lactose). Bottom 3 curves obtained with cells previously grown on glycerol. Induction by melibiose. (Top, no glycerol; middle, 17 μg/ml glycerol; bottom, 50 μg/ml glycerol.)

Glycerol itself is not inhibitory but must be metabolized to inhibit enzyme induction. This is indicated because other compounds available to supply energy have a similar inhibitory effect. Furthermore, glycerol, even at a concentration of 5 mg/ml, had no inhibitory effect on induction in the presence of uracil. Also, glycerol did not have an effect initially on induction of β-galactosidase in cells grown on glucose or lactate (which could not utilize glycerol).

The question as to why enzyme induction ceases after a short time in the absence of uracil and an external energy source is probably best answered in terms of appearance of an energy supply as enzyme formation proceeds. As soon as the inducer provides adequate energy, uracil will be used for growth and will no longer be available for induction. In support of this idea, it was observed that induction by lactose, which rapidly becomes available as an energy supply, was stopped sooner in the absence of uracil than induction by melibiose, which is not utilized for energy until an enzyme system for oxidation of melibiose becomes active at about 70 min (as measured by oxygen uptake). Further evidence regarding the role of energy supply in halting induction is presented in figure 4. Cells of mutant 6386 were grown on either glycerol or lactose plus minimal salts and uracil. They were then tested for ability to form β-galactosidase in the absence of uracil with lactose or melibiose, or both sugars, as inducers. The cells originally grown on lactose did not form more enzyme on lactose, or on melibiose plus lactose, but did make considerably more enzyme on melibiose alone. Presumably this occurred because the cells possessed an energy supply in the first and

second cases but not in the third. Furthermore, the final enzyme level reached by induction of lactose grown cells by melibiose was considerably higher than with glycerol grown cells. This shows that in glycerol grown cells the final concentration of enzyme was not limited by a saturation of the cells with β-galactosidase.

Demonstration of a requirement of nucleic acid components for β-galactosidase induction, by means of inhibitors. It would seem reasonable that mutants could provide a limited supply of purines and pyrimidines which would be adequate for enzyme induction only. If this were the case, competitive inhibitors of formation or incorporation of the bases should prevent the appearance of induced enzymes. For example, analogs of uracil might prevent enzyme synthesis if a trace of uracil is required, and their action should be reversed by uracil. Compounds tested and found inactive either as competitors with, or as substitutes for, uracil (for enzymic induction) at concentrations up to 2 mg/ml were: 2-thiouracil, 5-NH_2-uracil, 5-NO_2-uracil, 5-Cl-uracil, 5-Br-uracil, and 2-thiothymine. 5-I-uracil at 2 mg/ml and 2-thio-5-I-uracil at 1 mg/ml inhibited growth and enzyme induction, but the effect was not reversed by uracil or thymine. In some cases induction was inhibited but not growth; this effect, however, was not uniformly reproducible.

It was possible to inhibit induction by melibiose of β-galactosidase in mutant M45B4 (purineless) in the absence of an energy supply with 1.3 mg/ml of 2,6-diaminopurine. Addition of 26 μg/ml adenine almost completely eliminated the inhibition (table 1), demonstrating the effect to be competitive. It was previously shown that β-galactosidase formation does not occur with this mutant in glycerol-minimal salts (Pardee, 1954). Since casein hydrolyzate was required for good growth of this mutant, and this substance also provides energy, enzyme induction was not as extensive in the absence of adenine as in its presence. The results show that an intracellular supply of purines probably exists in this mutant in the absence of an energy supply.

Source of nucleic acid components for enzyme induction. Nucleic acid components might be provided either by partial breakdown of nucleic acids (turnover) or by slow synthesis (leaky mutant). Since only very small amounts of pyrimidines are required for enzyme induction, both possibilities seem admissible. Evidence presented below supports the "turnover" hypothesis. It is not possible to state whether nucleic acids are gradually broken down and resynthesized in all cells or whether death of a few per cent of the cells per hour provides material for the survivors.

TABLE 1

Effect of 2,6-diaminopurine on β-galactosidase formation

2,6-DAP	Adenine	β-Galactosidase Activity		
		30 min	60 min	90 min
−	−	7.5	12.0	9.0
+	−	0.5	1.0	3.5
−	+	25.0	32.0	45.0
+	+	16.0	36.0	42.0

E. coli, mutant M45B4 (purineless), was grown on minimal salts plus 30 μg/ml adenine, plus 0.1 per cent casamino acids plus glycerol. Cells were washed and tested for ability of melibiose to induce β-galactosidase in a medium containing 0.01 per cent casamino acids and minimal salts and in the presence or absence of 1.3 mg/ml 2,6-DAP or 26 μg/ml adenine. The numbers represent μg NPG hydrolyzed per minute per ml of original cells and are averages of two experiments.

To test the possibility that the mutants could make pyrimidines slowly by an alternative pathway, the incorporation of C^{14} into nucleic acids was measured. Mutant 6386 or mutant 550-460 was incubated 90 min with 1,3 C^{14} glycerol in minimal salts solution. The nucleic acids were isolated by TCA precipitation and were washed several times with TCA and alcohol. They were hydrolyzed and chromatographed by the method of Marshak and Vogel (1951), and the free bases were eluted and counted. The amount of C^{14} expected in nucleic acid pyrimidines was found as dihydroorotic acid or orotic acid in the two mutants, respectively (Yates and Pardee, *unpublished data*). Less than 0.1 per cent of this activity was found in the pyrimidines of the cell RNA, and about 0.3 per cent was found in the RNA purines. Incorporation of newly formed bases into nucleic acids is therefore negligible in these mutants.

Experiments on incorporation of $P^{32}O_4$ into pyrimidine requiring mutants 6386 or 550-460 in media minus and plus uracil provided evidence

TABLE 2

P^{32} uptake by mutants of Escherichia coli

Mutant	Medium			Counts Per Ml Original Solution/Min			
	Uracil	Glycerol	Melibiose	Acid soluble	RNA	DNA	P-lipid
6386	+	+	−	—	12,000	2,600	—
	−	+	−	—	420	230	1,200
550-460	+	+	−	4,400	15,000*		—
	−	+	−	1,900	540		—
	−	−	−	700	85		—
550-460	−	+	+	1,800	1,100*		—
	−	+	−	2,000	1,200*		—
	−	−	+	700	450		—
	−	−	−	600	50		—
550-460	+	+	+	—	8,700		5,900
	−	+	+	—	530		1,600
	−	−	+	—	170		25
	−	−	−	—	27		—

Cells were grown in minimal medium plus glycerol and uracil. They were washed and resuspended in medium containing P^{32}. Samples were taken at 1 hr, and the P^{32} incorporated into various fractions was determined. P-lipid is material extracted by alcohol but not by TCA.

* Phospholipids not removed in these experiments.

for a slow synthesis of nucleic acids in the absence of added pyrimidines. In the most detailed experiment there was about 8 per cent as much incorporation into DNA and 3 per cent as much into RNA in 1 hour in the pyrimidine deficient medium (containing glycerol) as in the complete medium (table 2). To show that the P^{32} was actually incorporated, the bacterial RNA was hydrolyzed and the nucleotides were separated by the method of Magasanik *et al.* (1950). Radioactive counts were associated with all the nucleotide spots. Counts in guanylic acid, the spot most free of background, was about 2 per cent of counts in guanylic acid of the control. In other experiments, 3 to 7 per cent as much P^{32} was found in nucleic acids of cells shaken in minimal salts plus glycerol as in the control culture. P^{32} was not incorporated appreciably into cells suspended in a medium lacking both energy supply and inducer. Cells provided with melibiose as inducer in the absence of uracil and glycerol incorporated one per cent as much P^{32} as did cells in a complete medium. It is not clear whether this uptake is a result of induction or occurs because melibiose provides a small amount of energy.

The experiments with P^{32} showed, first of all, that the mutants are able to synthesize RNA and DNA slowly in a medium lacking pyrimidines but containing glycerol. Second, the incorporation of P^{32} into the acid soluble pool is rapid under all conditions. Since phosphorus was not incorporated into nucleic acids in the absence of an energy supply and uracil, one obtains a differential labeling of the cell phosphorus in this way. Third, exchange of phosphorus between nucleic acids and acid soluble compounds ("turnover") is very slow, in agreement with earlier conclusions reached by other methods (Manson, 1953; Pardee, 1954). Fourth, considerable amounts of P^{32} were incorporated into phospholipids, which shows that synthesis of these compounds does not require the presence of pyrimidines.

Changes in nucleic acid composition indicative of partial breakdown are shown by analyses of mutant 6386 aerated in minimal salts-glycerol medium in the absence of pyrimidines. Between 1 and 4 hours at 37 C the number of cells remained substantially constant (±15 per cent) by both microscopic counts and viable counts. There was no increase of TCA precipitable ribose although deoxyribose increased slightly, and the material absorbing at 260 mμ decreased about 10 per cent over 4 hours. Instability of the RNA of *E. coli* has been observed by other investigators (Stephenson and Moyle, 1949).

The nucleic acids were analyzed by the method of Smith and Markham (1950). The total purines decreased by a few per cent, but pyrimidines (from RNA) decreased by about 13 per cent (table 3). These changes indicate a partial

TABLE 3

Changes in purines and pyrimidines of mutant 6386

Experiment	100 × Ratio of 5 hr Sample/1 hr Sample				
	Adenine	Guanine	Cytosine	Uracil	Total
I	98	96	88	86	93
II	95	96	84	91	92

Mutant 6386 was aerated in minimal salts plus glycerol. Samples were taken at 1 hr and 5 hr and were analyzed for purines and pyrimidines in the nucleic acids.

breakdown, leaving nucleic acids of a different composition.

DISCUSSION

Only a low concentration of uracil (less than 0.6 μg per ml) is required for enzyme induction by pyrimidine requiring mutants (figure 2). Since added uracil disappears quite slowly in the absence of an external energy source and since enzyme induction is favored relative to growth (figure 3), it seems very likely that the requirement for pyrimidines is met by their slow release from nucleic acids when an energy source is not present to reincorporate the bases into all nucleic acids.

These results are related to work of Gale and Folkes (1953) on protein and nucleic acid synthesis in *Staphylococcus aureus*. In this organism an increase of nucleic acid and protein occurs in the absence of added purines and pyrimidines. However, extensive synthesis required a supplement of nucleic acid components. Their results were explained in terms of a limited supply of bases in the cells.

Limited induced enzyme synthesis by purine or pyrimidine requiring strains of *Aerobacter aerogenes* in the absence of purines or pyrimidines, respectively (Ushiba and Magasanik, 1952), can also be explained in terms of a small reserve of bases which would be used for enzyme formation in the absence of an energy supply.

Since the present results are caused by inability of the mutants completely to eliminate available purines or pyrimidines, they do not conflict with the conclusion of the previous paper (Pardee, 1954) that RNA synthesis and protein synthesis are closely related. It may be pointed out that a requirement for RNA synthesis to permit protein synthesis does not necessarily mean that the two macromolecules are "woven" together. Another explanation might be that protein and RNA are made alternately, each molecule being useful for subsequent synthesis of one or a few molecules of the opposite kind.

It has often been observed that microorganisms can form induced enzymes in the absence of an external energy supply. In fact, most of the initial work on enzyme induction has been done under these "nongratuitous conditions". Although it is clear that many features of induction are more readily interpreted under gratuitous conditions (Monod *et al.*, 1952), it should be pointed out that factors other than the autocatalytic production of an energy supply appear to exist which differentiate gratuitous from nongratuitous conditions. Comparison of experiments under both types of conditions may therefore lead to better understanding of phases of induction such as interactions of formation of several enzymes or formation of "constitutive enzymes".

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SUMMARY

Certain strains of *Escherichia coli* which require nucleic acid components for growth were found to be capable of enzyme induction in the absence of these components only when no external energy source was present. The ratio of the rate of enzyme synthesis to protein synthesis was greatly increased in the absence of an external energy supply, compared to the ratio under "gratuitous conditions".

P^{32} is rapidly taken up in the acid soluble portion of the cells and by phospholipids, and slowly by nucleic acids, in the absence of necessary pyrimidines by pyrimidineless *E. coli* mutants, indicating, in combination with analytical data, a slow nucleic acid breakdown and resynthesis of new nucleic acids.

The results on induced enzyme synthesis may be explained in terms of a slow destruction of nucleic acid, making bases available which are reincorporated rapidly only when growth occurs. The observations are not in conflict with the concept that ribonucleic acid synthesis and protein synthesis are closely related.

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