JOURNAL OF BACTERIOLOGY
Vol. 88, No. 1, p. 99-104 July, 1964

ENZYMES OF THE PYRIMIDINE PATHWAY IN *ESCHERICHIA COLI*

I. SYNTHESIS BY CELLS AND SPHEROPLASTS

W. HERMAN TAYLOR AND G. DAVID NOVELLI

Biology Department, Portland State College, Portland, Oregon, and Biology Division, Oak Ridge National Laboratory, Oak Ridge, Tennessee[1]

Received for publication 24 February 1964

ABSTRACT

TAYLOR, W. HERMAN (Portland State College, Portland, Ore.), AND G. DAVID NOVELLI. Enzymes of the pyrimidine pathway in *Escherichia coli*. I. Synthesis by cells and spheroplasts. J. Bacteriol. **88**:99-104. 1964.—Upon release from repression, cells and spheroplasts of two mutants of *Escherichia coli* efficiently synthesized aspartate transcarbamylase and ornithine transcarbamylase, whereas only cells synthesized dihydroorotic dehydrogenase. Ethylenediaminetetraacetate treatment and sucrose incubation of cells were found to be responsible for the loss of dihydroorotic dehydrogenase synthesis. Spheroplasts required the addition of amino acids and an energy source for the synthesis of aspartate transcarbamylase. Uracil repressed synthesis of aspartate transcarbamylase in spheroplasts as well as in cells. Chloramphenicol inhibition and amino acid requirement for increased aspartate transcarbamylase activity in spheroplasts indicated de novo protein synthesis. *E. coli* 15, R185-482, and *E. coli* K-12, 496, were used to study the effect of carbon source and stimulation by orotate and dihydroorotate on synthesis of dihydroorotic dehydrogenase. Only *E. coli* 15, R185-482, showed any stimulation of dihydroorotic dehydrogenase synthesis. When glucose was the carbon source, orotate but not dihydroorotate stimulated; with glycerol as carbon source, dihydroorotate stimulated and orotate acted as a repressor. These results are discussed in terms of induction and pyrimidine supply to the cells.

Repression is control of enzyme synthesis by specific metabolites. Thus, the product of a biosynthetic pathway may regulate the level of enzymes which form it. Yates and Pardee (1957) showed that the pyrimidine pathway in *Escherichia coli* was repressible. Uracil-requiring mutants of *E. coli* contain low (repressed) enzyme levels after growth in uracil-supplemented medium. These repressed cells rapidly synthesize all enzymes of the pathway when transferred to fresh medium minus uracil. Enzyme levels are much higher in cells derepressed by pyrimidine starvation than in repressed cells. The rate of enzyme synthesis may be lowered at any time by addition of uracil.

We selected the pyrimidine pathway in *E. coli* with the ultimate goal of preparing spheroplasts and, subsequently, a cell-free system that would synthesize enzyme. Initial studies (Taylor, Tonomura, and Novelli, 1960) indicated that spheroplasts formed from repressed cells synthesized some enzymes with efficiency equal to that of cells, but did not synthesize dihydroorotic dehydrogenase. The inability of spheroplasts to form dihydroorotic dehydrogenase was studied further, and the results are reported here. Also, data on conditions controlling enzyme synthesis in cells and spheroplasts are presented.

MATERIALS AND METHODS

Mutants. E. coli K-12, mutant 496, obtained from L. L. Cavalli-Sforza, is blocked between orotic acid and uridylic acid. *E. coli* 15, R185-482 (a mutant of ATCC 9723), obtained from A. B. Pardee, requires arginine and uracil for growth and apparently is blocked at carbamyl phosphate synthesis (Thorne and Jones, 1963).

Cells and growth conditions. Cells were incubated with shaking at 37 C in a salts medium containing (in g per liter): KH_2PO_4, 2; K_2HPO_4, 7; $(NH_4)_2SO_4$, 1; Na_3 citrate, 0.5; $MgSO_4$, 0.1; $CaCl_2$, 0.01; $FeSO_4 \cdot 7H_2O$, 0.005; and glucose or glycerol, 5.0. Glucose was used as the carbon and energy source, except where glycerol is

[1] Operated by Union Carbide Corp. for the U.S. Atomic Energy Commission.

specifically listed (Table 4). Uracil (0.1 g per liter) and arginine (0.2 g per liter) were present in all growth media. Cells were released from repression by incubation in medium containing no uracil. Cultures (15 hr) were diluted tenfold with fresh medium and allowed to reach exponential growth; the cells were harvested by centrifugation, washed with growth medium minus uracil and carbon source, and resuspended in the appropriate medium for enzyme synthesis.

Enzyme synthesis. For enzyme synthesis, cells were shaken at 37 C in growth medium minus uracil, except where other conditions are stated. Spheroplasts were shaken at 37 C in synthesis medium containing: sucrose, 0.3 M; potassium phosphate (pH 7.0), 0.06 M; $MgCl_2$, 0.005 M; and glucose, 0.028 M. The amino acid mixture used contained the amino acids in the same relative concentrations as in *E. coli* protein (Roberts et al., 1957). Amino acid mixture (0.4 mg of amino acids per ml of incubation mixture) was added to all incubations given except the whole-cell incubations in Table 2. Samples removed initially and during the incubation were chilled to 0 C. Where indicated, a final concentration of 0.001 M sodium dihydroorotate (DHO) or sodium orotate was used.

Spheroplast formation. Spheroplasts were prepared by the lysozyme and ethylenediaminetetraacetate (EDTA) method of Rogers and Novelli (1959) except that 0.33 M sucrose was used. Efficiency of spheroplast formation was determined by phase microscopy and by lysis of spheroplasts with water.

Treatment of samples for enzyme assay. Cell suspensions were treated with toluene (0.05 ml per ml of cell suspension) for 5 min at room temperature, sedimented by centrifugation, and resuspended in water. Spheroplasts were sedimented from synthesis medium by centrifugation, and the pellets were resuspended in distilled water. Lysis occurred immediately, and assays were done on the clear viscous suspension. Enzyme synthesis by spheroplasts was always compared to the synthesis by a portion of culture from which the spheroplasts were prepared.

Enzyme assay and chemical determinations. Aspartate transcarbamylase was assayed by the method of Yates and Pardee (1957), except that ureidosuccinate was measured by the method of Koritz and Cohen (1954). The specific activity of repressed aspartate transcarbamylase in cells was approximately 1.0.

Ornithine transcarbamylase was assayed as described by Rogers and Novelli (1959). The specific activity of repressed ornithine transcarbamylase in cells was approximately 20.

Dihydroorotic dehydrogenase was usually assayed by ferricyanide reduction, as described in the next paper of this series (Taylor and Taylor, 1964). The activity in Table 1 was measured by the aerobic method of Yates and Pardee (1957). The specific activity of repressed dihydroorotic dehydrogenase was approximately 1.0 in mutant 496 cells and 3.0 in mutant R185-482 cells.

Orotidylic decarboxylase was measured by the method of Beckwith et al. (1962). Repressed (low) levels of enzyme activity were difficult to measure in toluene-treated cells or crude cell extracts owing to light scattering by particles. The specific activity of repressed orotidylic decarboxylase in cells was approximately 0.18.

A unit of enzyme is defined as the amount of enzyme which will produce 1 μmole of product (ureidosuccinate, orotate, uridylic acid, or citrulline) per hr under the conditions of the assay. Specific activity is listed as units of enzyme per milligram of protein.

Protein was measured by the Folin reagent (Lowry et al., 1951) with a bovine serum albumin standard.

RESULTS

The enzyme reactions we studied are listed below:

L-aspartate + carbamyl phosphate → carbamyl-L-aspartate + inorganic phosphate (1)

L-dihydroorotate → orotate + 2H (2)

orotidylic acid → uridylic acid + carbon dioxide (3)

L-ornithine + carbamyl phosphate → citrulline + inorganic phosphate (4)

Uracil represses formation of aspartate transcarbamylase (reaction 1), dihydroorotic dehydrogenase (reaction 2), and orotidylic decarboxylase (reaction 3), whereas arginine represses formation of ornithine transcarbamylase (reaction 4). The enzymes catalyzing reactions 1, 2, and 3 are involved in pyrimidine synthesis, whereas

TABLE 1. *Synthesis of enzymes by cells and spheroplasts of mutant 496*

Prepn	Time	Specific activity*		
		Ornithine transcarbamylase	Aspartate transcarbamylase	Dihydroorotic dehydrogenase
	min			
Cells	0	20.5	1.4	0.7
	90	36.5	13.7	5.0
Spheroplasts	0	42.5	2.8	0.9
	90	303.0	49.4	0.9

* Units of enzyme per milligram of protein.

TABLE 2. *Synthesis of enzymes by sucrose-grown cells and spheroplasts of mutant R185-482*

Prepn	Addition to medium*	Increase in specific activity during 60 min	
		Aspartate transcarbamylase	Dihydroorotic dehydrogenase
Cells	None	11.1	3.2
	Orotate	17.8	11.0
	Orotate + uracil	2.0	0.0
Spheroplasts	None	13.1	2.6
	Orotate	30.6	3.2
	Orotate + uracil	1.4	1.8

* Cells were cultivated on the salts medium supplemented with 0.33 M sucrose.

TABLE 3. *Requirements for aspartate transcarbamylase synthesis by spheroplasts of mutant 496*

Incubation medium	Increase in aspartate transcarbamylase specific activity in 60 min
Complete	80.4
Minus amino acids	0.5
Minus glucose	2.1
Minus amino acids and $(NH_4)_2SO_4$	0.4
Plus uracil	0.8
Plus chloramphenicol, 20 μg/ml	9.7
Plus chloramphenicol, 40 μg/ml	1.9

ornithine transcarbamylase is involved in arginine synthesis. Often, ornithine transcarbamylase synthesis was measured as an index of general synthetic ability of cells and spheroplasts.

Spheroplasts of mutants R185-482 and 496 were as efficient as cells in the synthesis of aspartate transcarbamylase and ornithine transcarbamylase, but only cells efficiently synthesized dihydroorotic dehydrogenase (Tables 1 and 2). The increase of ornithine transcarbamylase specific activity in cells was lower than in spheroplasts (Table 1), because arginine was in the incubation medium. Our finding that arginine repressed ornithine transcarbamylase synthesis in cells but not in spheroplasts was also reported by Rogers and Novelli (1959). In contrast, aspartate transcarbamylase synthesis was repressed by uracil in spheroplasts (Tables 2 and 3).

Conversion of cells to spheroplasts increased the specific activity of aspartate transcarbamylase and ornithine transcarbamylase twofold (Table 1). This twofold increase would be expected, because we found that half of the cell protein was lost during conversion of cells to spheroplasts. The specific activity of dihydroorotic dehydrogenase in spheroplasts was approximately the same as in cells, indicating a destruction of enzyme activity or a release of enzyme protein during spheroplast formation. No release of enzyme activity was measurable during spheroplast formation. In most experiments, there was little further loss of dihydroorotic dehydrogenase activity during incubation. Incubation of dihydroorotic dehydrogenase with lysozyme, in the same concentration as used for spheroplast formation, for 30 min did not affect enzyme activity. Cells to be converted to spheroplasts were grown in phosphate medium containing 0.3 M sucrose, because cells grown in phosphate medium without sucrose showed a 30- to 60-min lag in synthesis of dihydroorotic dehydrogenase. Spheroplasts formed from cells which had been grown in medium containing 0.3 M sucrose synthesized a small amount of dihydroorotic dehydrogenase (Table 2). No dihydroorotic dehydrogenase activity was found in the incubation medium after removal of spheroplasts for enzyme assays.

If cells were grown in medium containing 0.3 M sucrose and subsequently incubated in synthesis medium, dihydroorotic dehydrogenase formation was reduced to one-half that obtained when normally grown cells were incubated in synthesis

TABLE 4. *Effect of dihydroorotate, orotate, and carbon source on dihydroorotic dehydrogenase synthesis by cells*

Mutant	Carbon source	Addition	Increase during incubation*	
			Dihydroorotic dehydrogenase†	Protein
				mg/ml
R185-482	Glucose	None	0.0	0.00
		Orotate	30.0	0.32
		DHO	1.2	0.00
		DHO + orotate	30.6	0.34
	Glycerol	None	0.7	0.00
		Orotate	0.0	0.59
		DHO	33.2	0.29
		DHO + orotate	1.0	0.68
496	Glucose	None	9.2	0.17
		Orotate	9.8	0.15
		DHO	9.9	0.15
		DHO + orotate	10.0	0.17
	Glycerol	None	12.1	0.18
		Orotate	11.6	0.16
		DHO	11.2	0.18
		DHO + orotate	10.8	0.14

* Incubation time was 4.5 hr for R185-482 and 3 hr for 496.

† Expressed as units per mg of protein.

medium. In contrast to dihydroorotic dehydrogenase synthesis, aspartate transcarbamylase formation was stimulated by incubation in medium containing 0.3 M sucrose. EDTA treatment of cells did not affect the amount of aspartate transcarbamylase or dihydroorotic dehydrogenase synthesized, but the combined effects of growth in sucrose-containing medium, treatment with EDTA and incubation in synthesis medium gave the same extremely poor synthesis of dihydroorotic dehydrogenase as that obtained with spheroplasts. Growth of cells in medium containing higher phosphate concentration resulted in decreased synthesis of dihydroorotic dehydrogenase. At a concentration of 0.2 M phosphate, mutant R185-482 synthesized no dihydroorotic dehydrogenase, whereas mutant 496 synthesized half the amount of enzyme in 0.2 M phosphate as in 0.05 M phosphate.

Aspartate transcarbamylase synthesis in spheroplasts was dependent upon an energy source (glucose) and amino acid nitrogen (Table 3). Ammonium sulfate was not effective as a nitrogen source. Addition of uracil repressed enzyme formation, and little enzyme was formed in the presence of chloramphenicol.

Synthesis of dihydroorotic dehydrogenase and protein by mutant 496 was neither stimulated nor repressed by the addition of DHO or orotate to the incubation medium, regardless of carbon source used (Table 4). However, when glucose was the carbon source, enzyme synthesis in mutant R185-482 was stimulated by the addition of orotate. Addition of DHO had no effect when added alone or with orotate. An increase in protein was found only in the presence of orotate. When glycerol was the carbon source, the addition of DHO stimulated synthesis of dihydroorotic dehydrogenase, whereas the addition of orotate alone or in the presence of DHO completely repressed synthesis of dihydroorotic dehydrogenase. An increase in protein was found whenever orotate or DHO had been added, but the protein increase was much larger in the presence of orotate.

No synthesis of orotidylic decarboxylase, reaction 3, could be detected after incubation of spheroplasts, and no orotidylic decarboxylase activity could be found in the incubation medium after removal of spheroplasts for enzyme assay. Orotidylic decarboxylase synthesis in mutant R185-482 was affected by the same agents found to effect the synthesis of dihydroorotic dehydrogenase. Cells that synthesized high levels of dihydroorotic dehydrogenase also synthesized high levels of orotidylic decarboxylase. The specific activity of orotidylic decarboxylase was approximately tenfold less than that of dihydroorotic dehydrogenase.

DISCUSSION

The physiological state of cells has a marked effect on their ability to form dihydroorotic dehydrogenase. Maximal synthesis of dihydroorotic dehydrogenase was obtained only from cells harvested from cultures well into the exponential growth phase. Storage of mutant R185-482 cells in an ice bath for 60 min caused a marked decrease in dihydroorotic dehydrogenase synthesis, but synthesis of aspartate transcarbamylase and ornithine transcarbamylase was not inhibited by

this treatment. Therefore, cells were washed and resuspended as quickly as possible.

Synthesis of dihydroorotic dehydrogenase occurred only with a corresponding slow increase in protein, but synthesis of aspartate transcarbamylase and ornithine transcarbamylase proceeded in the absence of measurable protein synthesis. Spheroplasts synthesized no measurable protein under the conditions used, but efficiently synthesized aspartate transcarbamylase and ornithine transcarbamylase. It is possible that synthesis of dihydroorotic dehydrogenase is dependent on the growth of cell walls or membranes.

Because specific protein synthesis is regulated by the availability of unstable messenger ribonucleic acid (Brenner, Jacob, and Meselson, 1961), the amount of dihydroorotic dehydrogenase synthesized by mutant 496 would be limited by the endogenous pool of nucleotides. Thus, mutant 496, which cannot convert orotate to uridylic acid, synthesized a much lower level of dihydroorotic dehydrogenase than did mutant R185-482. Neither enzyme synthesis nor general protein synthesis in mutant 496 was effected by orotate or DHO. The addition of orotate to mutant R185-482, which is blocked at carbamyl phosphate synthesis, probably gave a continuous low pyrimidine supply, thus allowing the general protein synthesis found to be necessary for an increase in dihydroorotic dehydrogenase activity. Any manipulation of growth conditions increasing the rate of protein synthesis to a level near that obtained when uracil was present resulted in repression of dihydroorotic dehydrogenase synthesis; for example, orotate repressed glycerol-grown cells as efficiently as did uracil. Glucose-grown cells may be impermeable to DHO, because they synthesized neither general protein nor dihydroorotic dehydrogenase in the presence of DHO. Thus, DHO and orotate did not act as inducers of dihydroorotic dehydrogenase synthesis in either mutant, but these compounds stimulated enzyme synthesis in mutant R185-482 by providing a slow supply of precursor for pyrimidine biosynthesis. Orotate also gave a twofold stimulation of aspartate transcarbamylase synthesis in glucose-grown cells of mutant R185-482. Our findings on a single substrain of mutant R185-482 are in agreement with those of Yates and Pardee (1957). Their work was with various substrains of mutant R185-482. Those substrains that grew rapidly on a given uracil precursor showed the same repressed level of dihydroorotic dehydrogenase activity as found when uracil was present, whereas those substrains that grew slowly on a given precursor showed increased dihydroorotic dehydrogenase activity. They concluded that slow growth was probably due to low permeability of the precursor; thus, no intracellular repressor accumulated, because any precursor getting into the cell was immediately channeled into ribonucleic acid synthesis.

We found that arginine was required for synthesis of dihydroorotic dehydrogenase by mutant R185-482, indicating that protein must be synthesized to obtain dihydroorotic dehydrogenase activity. Because our experiments were designed primarily to measure dihydroorotic dehydrogenase synthesis (for which arginine was required), we did not test the effect of arginine on aspartate transcarbamylase synthesis. However, Thorne and Jones (1963) reported that arginine caused a 50% inhibition of aspartate transcarbamylase synthesis in mutant R185-482. Although arginine may not be required for aspartate transcarbamylase synthesis in cells, both amino acids and energy source were required for synthesis of this enzyme by spheroplasts. The chloramphenicol inhibition of increased aspartate transcarbamylase activity and the amino acid requirement indicate de novo enzyme synthesis, rather than activation of existing protein.

The inability of spheroplasts to synthesize dihydroorotic dehydrogenase and orotidylic decarboxylase may be due to damage of enzyme-forming sites on the cell membrane. Membrane preparations of *E. coli* were demonstrated by Spiegelman (1959) to be the major site of protein and nucleic acid synthesis. Suit (1962) found that the membrane fraction of *E. coli* prepared by penicillin treatment did not incorporate isotope into ribonucleic acid as rapidly as did membrane fractions obtained by other methods, indicating damage to the membrane during spheroplast formation. Our finding that synthesis of both orotidylic decarboxylase and dihydroorotic dehydrogenase was inhibited in spheroplasts may be correlated with the coordinate repression of these enzymes in *E. coli* (Beckwith et al., 1962). Even though the two enzymes are not localized on the same structures in the cell (Taylor and Taylor, 1964), they could be formed at the same site. There is no evidence yet that coordinately repressed enzymes are formed at the same site.

Our inability to detect dihydroorotic dehydrogenase synthesis in spheroplasts does not seem to

be due to assay problems, because addition of EDTA or sucrose to the assay mixtures did not affect enzyme activity.

It is probable that if any dihydroorotic dehydrogenase had been formed by spheroplasts it would be measured by our assay, because most of the dihydroorotic dehydrogenase activity in released cells was recovered in spheroplasts formed from them. Although McLellan and Lampen (1963) reported that acid phosphatase synthesized by yeast protoplasts was released into the incubation medium, we were unable to detect any increase of dihydroorotic dehydrogenase activity in either spheroplasts or incubation media after removal of spheroplasts for assay. Another possibility, which is difficult to test, is that spheroplasts form a dihydroorotic dehydrogenase which does not react with the aerobic or ferricyanide assay.

ACKNOWLEDGMENT

This investigation was supported in part by Public Health Service research grant GM 09955 from the National Institute of General Medical Sciences. Initial work was done while the senior author was a postdoctoral fellow of the Division of General Medical Sciences.

LITERATURE CITED

BECKWITH, J. R., A. B. PARDEE, R. AUSTRIAN, AND F. JACOB. 1962. Coordination of the synthesis of the enzymes in the pyrimidine pathway of *E. coli*. J. Mol. Biol. **5**:618–634.

BRENNER, S., F. JACOB, AND M. MESELSON. 1961. An unstable intermediate carrying information from genes to ribosomes for protein synthesis. Nature **190**:576–581.

KORITZ, S. B., AND P. P. COHEN. 1954. Colorimetric determination of carbamylamino acids and related compounds. J. Biol. Chem. **209**:145–150.

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

MCLELLAN, W. L., AND J. O. LAMPEN. 1963. The acid phosphatase of yeast: localization and secretion by protoplasts. Biochim. Biophys. Acta **67**:324–326.

ROBERTS, R. B., P. H. ABELSON, D. B. COWIE, E. T. BOLTEN, AND R. J. BRITTEN. 1957. Studies of biosynthesis in *Escherichia coli*. Carnegie Inst. Wash. Publ. 607.

ROGERS, P., AND G. D. NOVELLI. 1959. Formation of ornithine transcarbamylase in cells and protoplasts of *Escherichia coli*. Biochim. Biophys. Acta **33**:423–436.

SPIEGELMAN, S. 1959. Protein and nucleic acid synthesis in subcellular fractions of bacterial cells, p. 81–103. *In* Recent progress in microbiology, Symp. Intern. Congr. Microbiol. 7th, Stockholm, 1958. Charles C Thomas, Publisher, Springfield, Ill.

SUIT, J. C. 1962. Ribonucleic acid in a "membrane" fraction of *Escherichia coli* and its relation to cell-wall synthesis. J. Bacteriol. **84**:1061–1070.

TAYLOR, W. H., K. TONOMURA, AND G. D. NOVELLI. 1960. The synthesis of aspartyltranscarbamylase and dihydroorotic dehydrogenase by cells and protoplasts of *Escherichia coli*. Bacteriol. Proc., p. 148.

TAYLOR, W. H., AND M. L. TAYLOR. 1964. Enzymes of the pyrimidine pathway in *Escherichia coli*. II. Intracellular localization and properties of dihydroorotic dehydrogenase. J. Bacteriol. **88**:105–110.

THORNE, K. J. I., AND M. E. JONES. 1963. Carbamyl and acetyl phosphokinase activities of *Streptotoccus faecalis* and *Escherichia coli*. J. Biol. Chem. **238**:2992–2998.

YATES, R. A., AND A. B. PARDEE. 1957. Control by uracil of formation of enzymes required for orotate synthesis. J. Biol. Chem. **227**:677–692.