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Pyrimidine Pools and Macromolecular Composition of Pyrimidine-Limited *Escherichia coli*

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The growth rate of a pyrimidine-requiring strain was controlled by limiting the concentration of exogenous orotic acid. As the steady state, pyrimidine-limited growth rate was decreased, the intracellular pyrimidine pools and the total nucleic acid per unit mass of culture also decreased. The ratio of deoxyribonucleic acid to protein remained constant, whereas the ratio of ribonucleic acid to protein decreased 30% over a threefold variation in growth rate (50- to 150-min doubling times). The intracellular uridine triphosphate and cytosine triphosphate pools also decreased (although not coordinately), and the pyrimidine biosynthetic enzymes were derepressed. Cell size was unaffected by pyrimidine-mediated variation of the growth rate.

The macromolecular composition of bacterial cultures is known to vary as a function of the growth rate. Variations in the steady state growth rate caused by carbon, nitrogen, energy limitation, or the addition to the medium of amino acids, vitamins, and other growth-stimulating compounds result in several characteristic changes in the macromolecular composition of the culture. Generally in *Escherichia coli* and *Salmonella typhimurium* the deoxyribonucleic acid (DNA) and protein contents of the culture remain constant relative to mass, whereas the amount of ribonucleic acid (RNA) decreases with decreasing growth rate. The cell size and the amount of DNA per cell also decrease with decreasing growth rate (8, 12, 17).

In this paper we control the growth rate in a different way. We have been able to grow a pyrimidine-requiring strain of *E. coli* at various steady-state, pyrimidine-limited growth rates by simply varying the concentration of orotic acid supplied as source of pyrimidine. We then describe the relationships observed between pyrimidine-limited growth rate, pyrimidine pool size, and macromolecular composition.

MATERIALS AND METHODS

Bacterial media, strains, and growth conditions. A mutant strain of *E. coli* K-12, CYA288, requiring uracil and blocked at the aspartate transcarbamylase (ATCase, EC 2.1.3.2) step in pyrimidine biosynthesis (1), was obtained from A. B. Pardee. As shown in the results, some endogenous biosynthesis of pyrimidine occurred in this strain. A thymine-requiring (4 μg/ml) derivative (PD101) was isolated after trimethoprin selection (6).

The M63 minimal salts medium (16) was supplemented with glycerol (0.2%), Difco Casamino Acids (0.25%), and vitamin B_1 (5 μg/ml). All experiments were performed with exponentially growing (at 37 C for at least four to five divisions) cultures at a cell density of about 2×10^8 cells/ml. The results are expressed in terms of cellular mass, a unit of mass being equivalent to a cell density giving an optical density (OD) of 1.0 at 540 nm (about 4×10^8 cells/ml).

Cell labeling and fractionation. Five per cent trichloroacetic acid-insoluble material was collected on Millipore filters, washed with 5% cold trichloroacetic acid, dried, and counted. To assay acid-soluble pools, cells were collected on a filter, washed with five volumes of cold medium, and immediately resuspended in 5% trichloroacetic acid. After 30 min at 0 C, the sample was centrifuged for 10 min at 10,000 × *g*, and a portion of the supernatant fraction was counted. Fractionation of the nucleotides in this supernatant fluid was accomplished by first extracting with anhydrous diethyl ether 10 times and freeze-drying. The residue was resuspended in 0.1 ml of distilled water, and 5-μliter portions were used for chromatography and determination of total counts recovered. Separation of the nucleotides was carried out by the methods of Neuhard, Randerath, and Randerath (14), by using polyethylene imine-impregnated cellulose MN300 thin-layer chromatography plates (Brinkmann Instruments). Standard nucleotides were spotted along with the sample with an ultraviolet (UV) lamp to aid in the location of the nucleotides after chromatography. The nucleotide spots were cut out from the plate and counted

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In some cases, the chromatograms were covered with X-ray film, and the radioactivity on the chromatograms was shown to correspond with the UV-absorbing spots of the standards.

The specific activity of the pyrimidine nucleotides in the RNA was determined by first precipitating 20 ml of culture with 2 ml of 50% trichloroacetic acid. After 30 min at 0 C, the precipitates were centrifuged and washed five times in 5% trichloroacetic acid, dissolved in 1 ml of 0.3 N KOH, and incubated at 30 C for 20 hr to hydrolyze the RNA. The DNA and protein were then precipitated by addition of 3.4 ml of 10% trichloroacetic acid and removed by centrifugation. The supernatant fraction was extracted three times with anhydrous diethyl ether and chromatogrammed on a column (10 by 20 mm) of Bio-Rad AG1-X8 formate (200 to 400 mesh) previously washed with 2 M $NH_4^+HCOO^-$ (*p*H 2.9) and distilled water to reduce the background of UV-absorbing material. After applying the sample, the column was eluted with two 5-ml portions of 0.01 M $NH_4^+COO^-$ (*p*H 4.3), followed by five 3-ml portions of 0.06 M $NH_4^+COO^-$ (*p*H 2.9; reference 20). The 0.06 M fractions contained all the cytosine monophosphate (CMP) free of other contaminating nucleotides. The absorbance of these fractions was determined at 260 and 279 nm. The amount of CMP present was calculated by using an extinction coefficient at 279 nm of 12.2×10^3, which was determined experimentally at *p*H 2.9.

The DNA which was precipitated after RNA hydrolysis was centrifuged and washed twice with 5% trichloroacetic acid and dissolved in 0.8 ml of concentrated formic acid. After hydrolysis, the radioactive pyrimidine bases were chromatogrammed in a butanol (86%) and water (14%) solvent on Whatman 3MM paper (2). The amounts of radioactivity associated with the thymine and cytosine spots were determined by scanning with a Packard Strip scanner.

For counting most aqueous solutions, a standard dioxane-ethyl alcohol-naphthalene-1,4-bis-2-(5-phenyloxazolyl) benzene-2,5-diphenyloxazole solution was used. For aqueous solutions containing formate, Bray solution (3) was found most satisfactory. For nonaqueous materials, toluene scintillation fluid was used.

Enzyme assays. Enzyme assays for dihydroorotic acid dehydrogenase (DHOdehase, EC 1.3.3.1), orotidylic acid pyrophosphorylase (OMPppase, EC 2.4.2.10), and aspartate transcarbamylase were performed according to the procedures of Beckwith, Pardee, Austrian, and Jacob (1). Cell extracts were prepared by centrifuging, suspending the cells in 0.1 M phosphate buffer (*p*H 7.0), and sonicating the suspension for 3 min with a Branson cell disrupter equipped with a microprobe. Protein, RNA, and DNA were measured by conventional techniques (5, 11, 18).

RESULTS

Effect of orotic acid on growth rate. The growth rate of CYA288 in M63-glycerol-Casamino Acids medium was found to be dependent on the concentration of orotic acid supplied as the source of pyrimidine, and was always slower than in cultures supplied with uracil as a source of pyrimidine. The growth rate when uracil was supplied was 1.2 u (doublings per hour) and independent of the concentration of uracil supplied (until the supply was exhausted), whereas the growth rate in orotic acid media varied from 1.15 to 0.4 u. The growth curves of a series of cultures supplemented with either uracil (20 μg/ml) or orotic acid (5 to 100 μg/ml) are shown in Fig. 1. The number of cells per unit mass of culture was determined with a Coulter counter, and was found to be equal at all growth rates in media supplemented with uracil or orotic acid. A 30 to 40% decrease in cell size would be expected over this range of growth rates, limited by carbon-energy source (12).

Endogenous pyrimidine biosynthesis. When cells were grown on limiting concentrations of orotic acid, a considerable proportion of the pyrimidine incorporated into cellular nucleic acid was of endogenous origin. An experiment was designed to determine the proportion of exogenous pyrimidine incorporated into cellular nucleic acids at different growth rates. The ratio of the specific activity of CMP in RNA to the specific activity of exogenous orotic acid was determined as a function of growth rate (Fig. 2). At growth rates faster than 0.8 u, 80 to 90% of the pyrimidine incorporated into cellular RNA was derived from the orotic acid in the growth medium. At slower growth rates the amount of exogenous pyrimidine incorporated decreased rapidly, until, at 0.4 u, only about 20% of the total cellular pyrimidine was derived from exogenous orotic acid. Extracts of the mutant strain show no ATCase activity even under conditions normally resulting in derepression of the enzyme. Growth in medium containing ^{14}C-aspartic acid, a normal precursor of uridine monophosphate (UMP), results in efficient labeling of the endogenously synthesized pyrimidine.

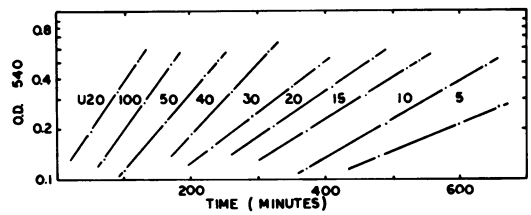

FIG. 1. *Growth curves. The optical density was monitored during exponential growth in cultures supplied with 20 μg of uracil (designated "U-20" in the figure) per ml and 100, 50, 40, 30, 20, 15, 10, and 5 μg of orotic acid per ml (listed above each curve). The growth curves have been offset on the time axis for purposes of illustration.*

To determine whether the cytosine and thymine incorporated into DNA were in radioisotopic equilibrium at different growth rates, the DNA from labeled cultures was hydrolyzed with formic acid, the free bases were separated chromatographically, and the radioactivities of the thymine and cytosine were determined (Table 1). As in the case of RNA, the amount of radioactivity in thymine and cytosine of DNA decreased as the growth rate decreased, because of dilution by the endogenously synthesized pyrimidine at the slower growth rates. The ratio of thymine to cytosine in *E. coli* DNA is 1.02 (9), and, as is evident from Table 1, the ratio of the radioactivity of thymine to the radioactivity of cytosine was approximately equal to this value at all growth rates. In line with this result, we shall assume for these long-term labeling experiments that, at each steady-state growth rate, the major components of the pyrimidine pool have the same specific activity as that of the cytosine in the RNA.

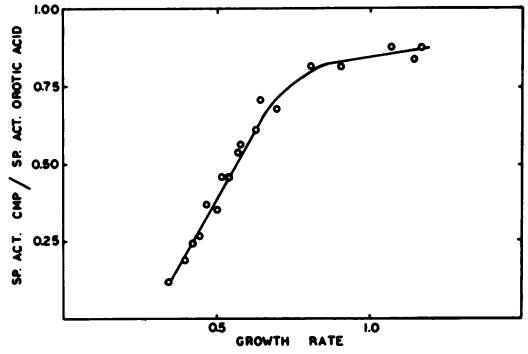

FIG. 2. *Radioactivity incorporated into CMP of RNA. Cultures were supplemented with orotic acid-6-^{14}C (30.8 μc/mmole; Schwarz BioResearch Corp.) at concentrations ranging from 5 to 100 μg/ml. The ratio of the specific activity of CMP isolated after RNA hydrolysis to the specific activity of the exogenous orotic acid is illustrated as a function of growth rate (doublings per hour).*

TABLE 1. *Incorporation of labeled orotic acid into pyrimidine bases of deoxyribonucleic acid*

Growth rate (u)	Units of relative radioactivity[a]	
	Thymine	Cytosine
1.17	270	304
0.70	274	286
0.64	219	228
0.57	178	187
0.51	145	146
0.43	67	72

[a] Units of radioactivity represent the area under peaks obtained by scanning the chromatograms. A unit of activity is equivalent to approximately 50 disintegrations per min. The deoxyribonucleic acid preparations were isolated after the alkaline hydrolysis of the trichloroacetic acid precipitates from cultures described in the legend to Fig. 2.

DNA, RNA, protein, and trichloroacetic acid-soluble pools. Orcinol, diphenylamine, and Folin assays conducted on trichloroacetic acid precipitates from cells grown at different growth rates indicated that the ratio of DNA to protein remained constant over the growth rates studied, but that the ratio of RNA to protein decreased from 0.325 at 1.1 u to 0.225 at 0.45 u (Fig. 3). In addition, a thymine auxotroph, derived from strain CYA288, was grown in the presence of ^{14}C-thymine at growth rates regulated by orotic acid concentrations in the medium. The radioactivity incorporated into the acid-precipitable material per unit mass remained constant and independent of the growth rate (Fig. 4). We have also followed the incorporation of ^{14}C-orotic acid into trichloroacetic acid-insoluble material, and,

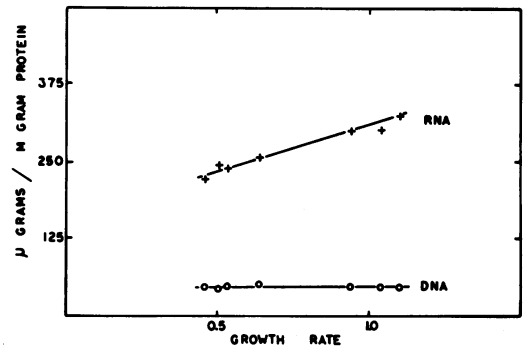

FIG. 3. *Ratios of RNA to protein and DNA to protein in steady-state, pyrimidine-limited cultures.*

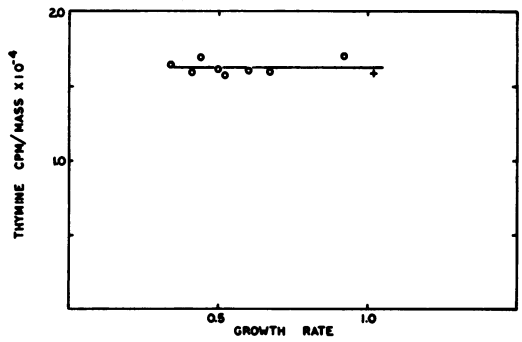

FIG. 4. *Radioactivity incorporated into DNA. Steady state, pyrimidine-limited cultures were grown in media containing 4 μg of ^{14}C-thymine per ml (31.4 μc/mmole, New England Nuclear Corp.). Orotic acid concentrations ranged from 5 to 100 μg/ml* (○), *and the uracil concentration was 20 μg/ml* (+).

when the results are corrected for endogenous pyrimidine biosynthesis (the data of Fig. 2), the resulting curve of total nucleic acid per unit mass agrees well with the data of Fig. 3.

The incorporation of orotic acid-6-^{14}C into the intracellular trichloroacteic acid-soluble pool is plotted in the lower curve of Fig. 5 as a function of growth rate. The upper curve illustrates the total intracellular pyrimidine pool as a function of growth rate, assuming that the specific activity of the pyrimidine pool is the same as the specific activity of the cytosine in RNA; the upper curve was obtained by dividing the lower curve by the curve in Fig. 2.

The effect of the washing procedure on the stability of the intracellular pools, following the collection of the cells on the filters and prior to the extraction with trichloroacetic acid, was examined. While still on the Millipore filters, radioactive cell samples were washed with from 0 to 15 volumes of ice cold medium in 1-ml portions. The amount of radioactivity recoverable in the acid supernatant fraction after washing with two or more 1-ml portions of cold medium remained approximately constant. A second control, involving the use of 5% perchloric acid in place of 5% trichloroacetic acid as the extracting solution, failed to produce any quantitative differences in the intracellular pyrimidine pools at several different growth rates.

The fractions of the total trichloroacetic acid-soluble radioactive pool labeled with orotic-6-^{14}C acid present as uridine triphosphate (UTP) and cytosine triphosphate (CTP) are shown in Fig. 6 as a function of growth rate. The percentage of the pool as UTP decreased drastically, whereas the percentage of the pool as CTP remained approximately constant as the growth rate decreased. The recoveries of radioactivity after ether extraction and lyophilization were approximately 80%.

The intracellular concentrations of UTP and CTP (moles per unit mass) as a function of growth rate can be obtained on the assumption that the specific activities of UTP and CTP are the same as the specific activity of cytosine in RNA: each of the curves of Fig. 6 (fractions of total trichloroacetic acid-soluble counts as UTP and CTP) is multiplied by the lower curve of Fig. 5 (total trichloroacetic acid-soluble counts per unit mass) and divided by the curve of Fig. 2 (fraction of cytosine in RNA labeled by exogenous supply). This is mathematically equivalent to multiplying the curves of Fig. 6 by the upper curve of Fig. 5 (total pyrimidine pool). It is apparent without presenting the curves that both the UTP and CTP pools decline with decreasing growth rate, and that the response of the UTP pool to pyrimidine limitation is much more severe than is the response of the CTP pool.

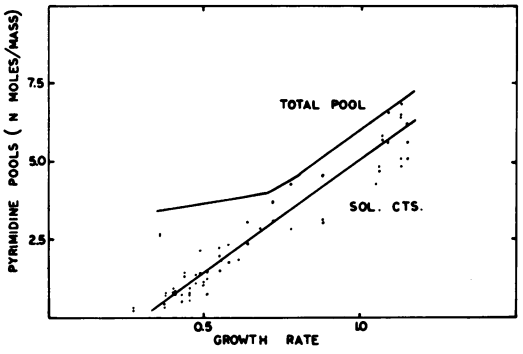

FIG. 5. *Radioactivity incorporated into trichloroacetic acid-soluble material. Cultures were supplemented with orotic acid-6-^{14}C (522 μc/mmole) at concentrations ranging from 5 to 100 μg/ml of culture. The upper curve is corrected for endogenous biosynthesis and represents the total pool.*

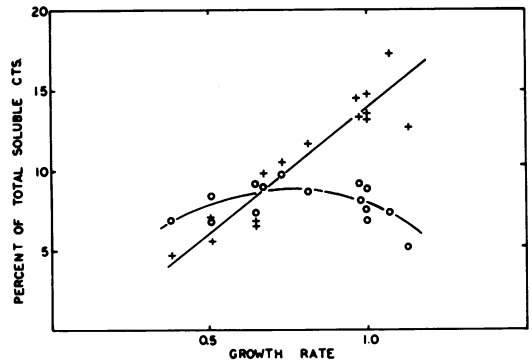

FIG. 6. *Percentage of labeled pool as UTP and as CTP. Cultures were labeled with orotic acid-6-^{14}C (4,300 μc/mmole). The pools were extracted from 5-ml portions and chromatogrammed. The percentage of total counts extracted appearing as UTP (+) and CTP (○) are plotted as a function of growth rate.*

A control to monitor the stability of the nucleotide triphosphates during extraction and concentration was conducted by adding ^{14}C-UTP to a trichloroacetic acid extract prepared from unlabeled cells. After ether extraction, lyophilization, and chromatography, more than 98% of the radioactivity on the chromatograms was associated with the UTP spot.

Effects of pyrimidine limitation on enzyme synthesis. The enzymatic activities of two of the pyrimidine biosynthetic enzymes were measured in extracts prepared from cultures grown on media containing orotic acid (10 to 100 μg/ml) as the source of pyrimidine. The relationships between the extent of derepression of DHOdehase and OMPppase as a function of growth rate are

shown in Fig. 7. The two enzymes show slightly different responses to pyrimidine limitation and are probably not strictly coordinate.

Intracellular pools and macromolecular composition of cultures grown on media containing uracil. Table 2 compares the results obtained with cultures grown on uracil (20 μg/ml) and cultures grown on orotic acid (100 μg/ml). Cultures supplied with uracil grew about 3 to 7% faster than cultures supplied with 100 μg of orotic acid per ml, but the ratios of DNA (diphenylamine) to protein (Folin) and RNA (orcinol) to protein were approximately equal in the two cultures. Isolation of CMP from the RNA after radioactive labeling with uracil-*2*-^{14}C showed that the rate of endogenous pyrimidine biosynthesis was the same as in the case of growth on 100 μg of orotic acid-*6*-^{14}C. The major differences between growth on uracil and growth on orotic acid were reflected in the concentration and composition of the intracellular pyrimidine pools. The intracellular pyrimidine pools (total labeled pool, UTP pool and CTP pool) were smaller in cultures supplemented with uracil than in cultures supplemented with 100 μg of orotic acid per ml (Table 2).

The specific activities of DHOdehase and OMPppase in extracts from cultures grown on 20 μg of uracil per ml were approximately the same as from cultures grown on 100 μg of orotic acid per ml.

DISCUSSION

We have been able to control the growth rate of a particular, pyrimidine-requiring strain of *E. coli* by simply varying the concentration of orotic acid supplied as pyrimidine. Orotic acid, unlike uracil, is apparently taken up by the cell very inefficiently.

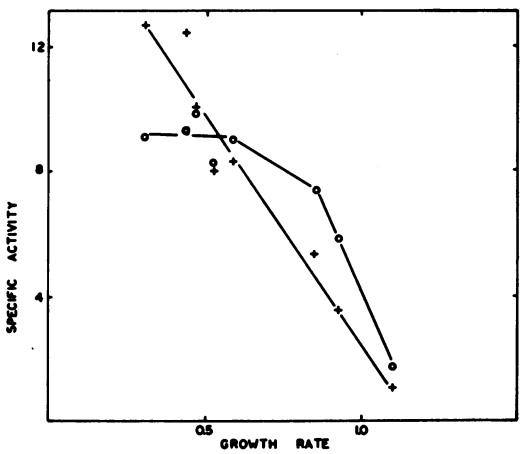

FIG. 7. *Derepression of DHOdehase* (+) *and OMPppase* (○). *Specific activities are expressed in units of enzyme per milligram of protein.*

TABLE 2. *Macromolecular composition of cultures grown on 20 μg of uracil per ml or 100 μg of orotic acid per ml*

	Pyrimidine source	
	Uracil	Orotic acid
RNA/protein (mg/mg)[a]	0.325	0.302
DNA/protein (mg/mg)[a]	0.0470	0.0469
Labeled trichloroacetic acid-soluble pool (nmoles/unit mass)[b]	3.44 ± .29	5.88 ± .93
Per cent of labeled pool as UTP[c]	11.7	14.9
Per cent of labeled pool as CTP[c]	6.45	5.25
UTP pool (nmoles/mass)[d]	0.45	1.02
CTP pool (nmoles/mass)[d]	0.25	0.36

[a] Orcinol, diphenylamine, and Folin assays for ribonucleic acid (RNA), deoxyribonucleic acid (DNA) and protein on trichloroacetic acid precipitates.

[b] Average values from seven independent measurements of the exogenously labeled intracellular pyrimidine pools. The calculated standard deviation of the seven measurements is included.

[c] Determined by thin-layer chromatography. Orotic acid data were averaged from two experiments, and uracil data from three experiments.

[d] Uridine triphosphate (UTP) [or cytosine triphosphate (CTP)] pool = per cent of labeled pool as UTP (or CTP) × labeled trichloroacetic acid-soluble pool per fraction of cytosine in RNA labeled by exogenous supply.

Limited but constant growth rates are set by rather high concentrations of exogenous orotic acid. (Other pyrimidine-requiring strains of *E. coli* we have tested were unable to grow at all on orotic acid as source of pyrimidine.) Uracil, on the other hand, is taken up very efficiently and does not limit growth until the medium is nearly completely depleted.

The variation in growth rate mediated by the supply of orotic acid was paralleled by a variation in the pyrimidine pool sizes (including, in particular, the UTP and CTP pool sizes). As expected, at the lowest growth rates, the pools appeared to be considerably smaller than those observed with uracil-grown cells. At 100 μg of orotic acid per ml, however, where the growth rate was almost equal to that found with uracil, the pyrimidine pools appeared to be considerably larger than those found in uracil-grown cells. The latter result may be related to the fact that the intracellular pool in uracil-grown cells cannot be expanded by increas-

ing the concentration of uracil (15). Neuhard has recently presented evidence for a feedback inhibition of UMP-pyrophosphorylase in *S. typhimurium*, by UTP or some other uracil-containing nucleotide, which restricts the accumulation of intracellular pyrimidine nucleotides in uracil-grown cells (13).

We have also observed that the intracellular concentrations of UTP and CTP do not drop coordinately as the pyrimidine-limited growth rate is decreased: the UTP pool is depressed much more rapidly than is the CTP pool. We have evidence that indicates that nucleotides containing cytosine (CMP, cytosine diphosphate, deoxycytosine diphosphate, and deoxycytosine triphosphate) closely resemble CTP in their response to pyrimidine limitation, whereas uracil and thymine nucleotides (deoxythymidylate, deoxythymidine diphosphate, deoxythymidine triphosphate, UMP, and UDP) resemble UTP. However, these experiments are technically difficult because of the small sizes of these pools relative to the total pyrimidine pool. Long and Pardee (10) have shown that the enzyme responsible for conversion of UTP to CTP, CTP synthetase (E.C. 6.3.4.2), is inhibited allosterically by its product, CTP, and is activated by UTP and guanosine (GTP).

When the growth rate of *E. coli* or *S. typhimurium* is controlled by carbon, nitrogen, or energy limitation, or by the presence of amino acids, nucleosides, vitamins, or other growth-stimulating compounds, several characteristic changes in the macromolecular composition of the cell occur (8, 12, 17). The DNA to protein ratio remains almost constant, whereas the RNA to protein ratio decreases with decreasing growth rate. We have followed the same parameters as a function of growth rate in pyrimidine-limited cultures and obtained essentially identical results. As shown in the accompanying paper (4), however, the relative proportions of transfer RNA and ribosomal RNA are markedly different in pyrimidine-limited cultures as compared with cultures limited by the carbon and energy source.

Our experiments on the derepression of DHOdehase and OMPppase suggest that the syntheses of these enzymes are not coordinately controlled at intermediate levels of pyrimidine limitation. Beckwith, Pardee, Austrian, and Jacob (1) have reported that during starvation for uacil in pyrimidine-requiring strains, these enzymes (as well as two others) were derepressed coordinately. The genes specifying DHOdehase and OMPppase are not closely linked, however (19).

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