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Synchronously Dividing Bacterial Cultures

I. Synchrony Following Depletion and Resupplementation of a Required Amino Acid in *Escherichia coli*

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ABSTRACT

MATNEY, THOMAS S. (The University of Texas M. D. Anderson Hospital and Tumor Institute, Houston, Texas), AND JOAN C. SUIT. Synchronously dividing bacterial cultures. I. Synchrony following depletion and resupplementation of a required amino acid in *Escherichia coli*. J. Bacteriol. **92**:960–966. 1966.—A procedure was developed for phasing large-volume cultures of *Escherichia coli* K-12 with regard to cell division. The method consists of permitting the bacteria to exhaust a growth-limiting supply of a required amino acid, starving the culture, resupplementing with an excess of the amino acid, and following the ensuing growth by usual counting procedures.

Inhibition of protein synthesis in *Escherichia coli* K-12 by deprivation of a required amino acid results in the ultimate arrest of both ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) synthesis. The depletion method for inducing synthrony was based on the assumption that, as the supply of a required amino acid is exhausted, DNA and RNA synthesis will be arrested in all cells at the amino acid-critical steps, resulting in an alignment of the population.

MATERIALS AND METHODS

Bacteria. The derivatives of *E. coli* K-12 employed in this investigation, and their properties, are listed in Table 1. Hfr derivatives were stocked in sealed stab tubes of Difco Nutrient Broth with 0.5% NaCl and 0.75% agar. Working stocks of non-Hfr cultures were maintained on nutrient agar slants.

These cultures were selected for their wide variation in capacity to synthesize nucleic acids after withdrawal of a required amino acid. The amount of RNA synthesized by *E. coli* K-12 derivatives was shown by Stent and Brenner (8) to be controlled by a single genetic locus (termed *RC* for RNA Control). The wild-type allele, RC^{st}, was found to exert a stringent control; i.e., <10% increase in RNA is observed during amino acid starvation of an RC^{st} organism. The mutant allele, RC^{rel}, relaxes the control, permitting an increase of >60% during amino acid starvation. Although both the P4X and the HfrH donors were originally isolated from the same F^+ *met-161*$^-$ RC^{rel} mutant of Lederberg (W6), the HfrH used in this study was prototrophic as obtained from the Cold Spring Harbor collection, and, after ultraviolet induction of a different methionine mutation, *met-23*$^-$, proved to be RC^{st} as well. The family of derivatives carrying the *his-323*$^-$ mutation are unique in their low capacity to synthesize DNA during starvation for histidine (Suit, Goldschmidt, and Matney, Bacteriol. Proc., p. 14, 1965).

Medium. The minimal medium (MM) of Haas and Doudney (2) contained K_2HPO_4, 7.0 g; KH_2PO_4, 3.0 g; Na citrate·$2H_2O$, 0.5 g; $MgSO_4 \cdot 7H_2O$, 0.1 g $(NH_4)_2 \cdot SO_4$, 1.0 g; and glucose, 2 g (in 1 liter of demineralized water). The glucose was autoclaved separately. Since some strains required thiamine, it was routinely added to the medium at a final concentration of 5 μg/ml. Full growth requirements of the strains were met by the following supplements: histidine, 100 μg/ml; methionine, tryptophan, and arginine, 20 μg/ml; and thymine, 2 μg/ml for strains carrying mutations 156 and 334, and 20 μg/ml for mutation 88.

Isolation of thymineless mutants. Thymine-requiring auxotrophs were derived from Hfr P4X *met-161*$^-$, Hfr G6 *his-323*$^-$, and F^- *his-323*$^-$ by the method of Nishioka and Eisenstark (*personal communication*). MM was supplemented with the required amino acid, thiamine, 1% glucose, 5 μg/ml of thymidine, 200 μg/ml of aminopterin, and 3 μg/ml of *N*-methyl-*N*′-nitrosoguanidine (Aldrich Chemical Co., Milwaukee, Wis.); 1 ml of this medium inoculated with a small loopful of an overnight culture was incubated at 37 C with aeration. After 2 days, turbidity appeared; a loopful was streaked onto hard minimal plates supplemented with glucose, thiamine, the required amino acid, and 2 μg/ml of thymidine. The plates were incubated at 37 C overnight. Of the colonies that developed, those that were translucent and irregularly

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granular proved to be thymineless. Several colonies were picked and restreaked for purification. Isolates were marker-checked and, in the case of the donors, checked for fertility by cross-streaking with a suitable recipient on an MM agar plate. The isolates that proved satisfactory were designated Hfr P4X *met-161⁻ thy-88⁻*, Hfr G6 *his-323⁻ thy-87⁻*, and F⁻ *his-323⁻ thy-329⁻*. Thymine (10 μg/ml or more) was required for optimal growth in liquid media. Derivatives with low requirements (2 μg/ml or less) were isolated from the latter two auxotrophs by the method of Harrison (3). They were designated Hfr G6 *his-323⁻ thy-156⁻* and F⁻ *his-323⁻ thy-334⁻*, respectively.

Preparation of inocula. The amino acid-requiring mutant was grown overnight in MM with appropriate supplements. It was diluted into fresh MM to an optical density (OD) of 0.05 at 660 mμ, in a Bausch and Lomb Spectronic-20 colorimeter, and growth was continued with aeration at 37 C until an OD of 0.5 was attained. Small-volume cultures (50 ml) were grown in 300-ml Erlenmeyer flasks fitted with standardized side-arm tubes to facilitate photometric measurements; incubation took place in a (37 C) waterbath-shaker. Large-volume cultures (1 liter) were grown in 2-liter Erlenmeyer flasks with forced aeration by sparging. The cells were harvested and washed twice with single-strength MM salts, by use of membrane filtration for small-volume cultures and centrifugation at 4 C for large cultures; the washed cells were then suspended in one-fifth volume of wash liquid. The resulting cell suspensions were stored in the refrigerator and used as inocula for 1 week.

Synchronization. The inoculum suspension of cells was diluted 50-fold (to an OD of 0.05) into MM supplemented with 1 μg/ml of the required amino acid. Growth during depletion of the amino acid was followed turbidimetrically. After depletion, incubation at 37 C was continued for 3 hr past the final small inflection in OD before addition of the usual amino acid growth supplement (100 μg/ml of histidine; 20 μg/ml of the other amino acids). Subsequent growth was measured by direct cell counts in a Petroff-Hauser counting chamber. Samples (0.5 ml) were pipetted into 4.5 ml of chilled diluent containing 0.8% NaCl and 5×10^{-3} M NaN_3. This preservative was found to be necessary if the cells were held 1 day or longer at 4 C before counting; both phenol and formalin were found to be ineffective in preventing cell division in refrigerated samples. The samples were thoroughly mixed by holding the tubes against a Vortex mixer, and a 10-lambda capillary pipette was used to load the counting chamber to assure uniformity of sample volume. For each sample, cells in 10 fields of 20 small grid squares each were counted. This total count, multiplied by 10^6, gives the number of bacteria per milliliter of culture.

The direct counting procedure was preferred, since the uniform elongation of cells and the formation of doublets prior to division were readily observed. There is, however, no substitute for experience with the direct counting procedure. The results in Fig. 1a and 2 reflect the variation encountered in early experiments. Later, 10 separate measurements of a sample yielded counts ranging from 139 to 146, with an average of 143 and a calculated standard deviation of 2.29.

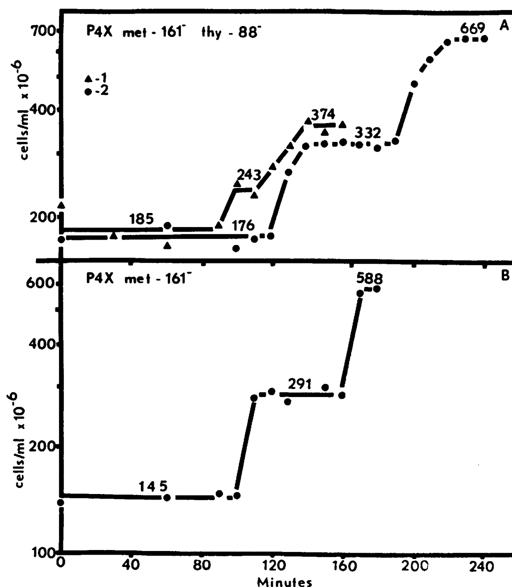


FIG. 1. *(A) Phased growth of a thymine-deficient mutant of Hfr P4X. Curve 1 was obtained by resupplementing with 20 µg of methionine per ml after 1 hr of starvation, and curve 2 by resupplementing after 3 hr. The numbers above each plateau represent the average plateau values. (B) Phased growth of the parental Hfr P4X.*

Measurement of protein, RNA, and DNA. Samples (10 ml) of culture were removed at specified time intervals for chemical analyses. The samples were extracted by the method of Ogur and Rosen (7) and analyzed for DNA by the diphenylamine method of Burton (1). RNA was determined by the method of Visser and Chargaff (9), and protein by the method of Lowry et al. (4).

RESULTS

The time of incubation intervening between the depletion of 1 μg of amino acid per ml and resupplementation with an excess of amino acid was shown to have an effect upon the subsequent division patterns. The time of depletion was operationally defined as the time at which the last small increment in OD appeared (see Fig. 4). In Fig. 1A, the division pattern shown by curve 1 was obtained when a depleted culture of P4X-88 had been resupplemented after 1 hr of starvation; in curve 2, 3 hr of starvation ensued prior to resupplementation with 20 μg of methionine per ml. The freshly depleted culture appeared to be composed of two distinct populations, each demonstrating a separate schedule of subsequent cell division. With adequate starva-

TABLE 1. *Characterization of Escherichia coli K-12 derivatives*

Strain	Mating type[a]	Nutritional mutations[b]	Generation time	Capacity to synthesize[c]	
				RNA	DNA
			min		
G6	Hfr	*his-323⁻*	62	<10	<15
G6-156	Hfr	*his-323⁻ thy-156⁻*	64	<10	<15
334	F⁻	*his-323⁻ thy-334⁻*	68	<10	<15
P4X	Hfr	*met-161⁻*	57	>60	>40
P4X-88	Hfr	*met-161⁻ thy-88⁻*	70	>60	>40
HfrH	Hfr	*met-23⁻*	53	<10	<40
1021	F⁻	*argG-1021⁻ B₁⁻*	50	<10	<40
444	F⁻	*try-444⁻*	60	<10	<40

[a] Hfr chromosomal donation patterns have been described (5).

[b] Symbols correspond to histidine (*his*), thymine (*thy*), methionine (*met*), arginine (*arg*), thiamine (B_1), and tryptophan (*try*); the number following the symbol refers to the specific mutation.

[c] Numbers represent percentage increase in nucleic acid after withdrawal of required amino acid from rapidly growing culture.

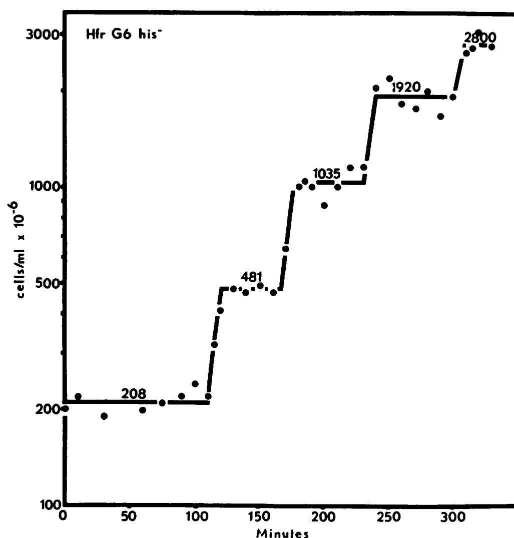


FIG. 2. *Phased growth of Hfr G6. The inoculum for this early experiment was prepared by washing cells from an overnight culture twice with saline (0.8% NaCl) and diluting to an OD of 0.05 in depletion medium. The variation in direct counts reflects lack of experience and refinement of techniques.*

tion, the two division patterns reinforced one another. Since the 3-hr starvation time seemed to assure complete phasing of cell division in all cultures tested, it was adopted as standard procedure for subsequent studies.

The presence of the mutation, *thy-88⁻*, in P4X increased the time required for the OD to double in liquid MM from 57 to 70 min (Table 1). A similar effect was noted when the times of the phased division cycles were compared (Fig. 1B, 60 min for the parental P4X; curve 2 in Fig. 1A, 70 min for the *thy-88⁻* mutant). In contrast, the mutations, *thy-156⁻* and *thy-334⁻*, did not increase the generation time appreciably (Table 1).

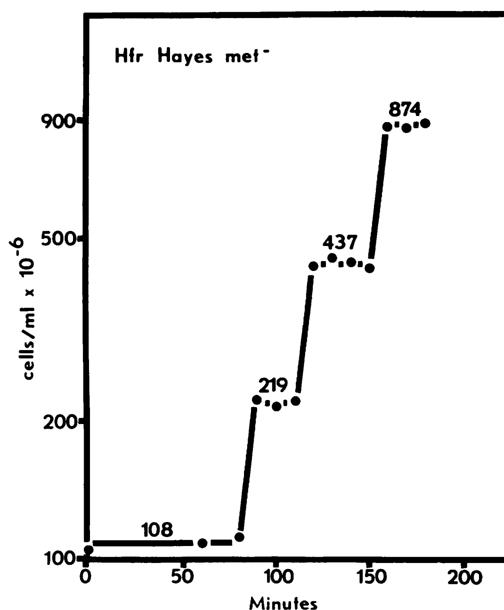


FIG. 3. *Phased growth of Hfr Hayes.*

In Fig. 2, the phased growth of G6 was followed to completion (2.8×10^9 cells per milliliter). Three full division cycles were accomplished, followed by a final partial cycle. The length of the average division cycle, 63 min, is in agreement with the generation time, 62 min (Table 1).

The phased division pattern obtained with

Table 2. PHASING OF DNA⁻/RC^st DERIVATIVES OF *E. coli* K12

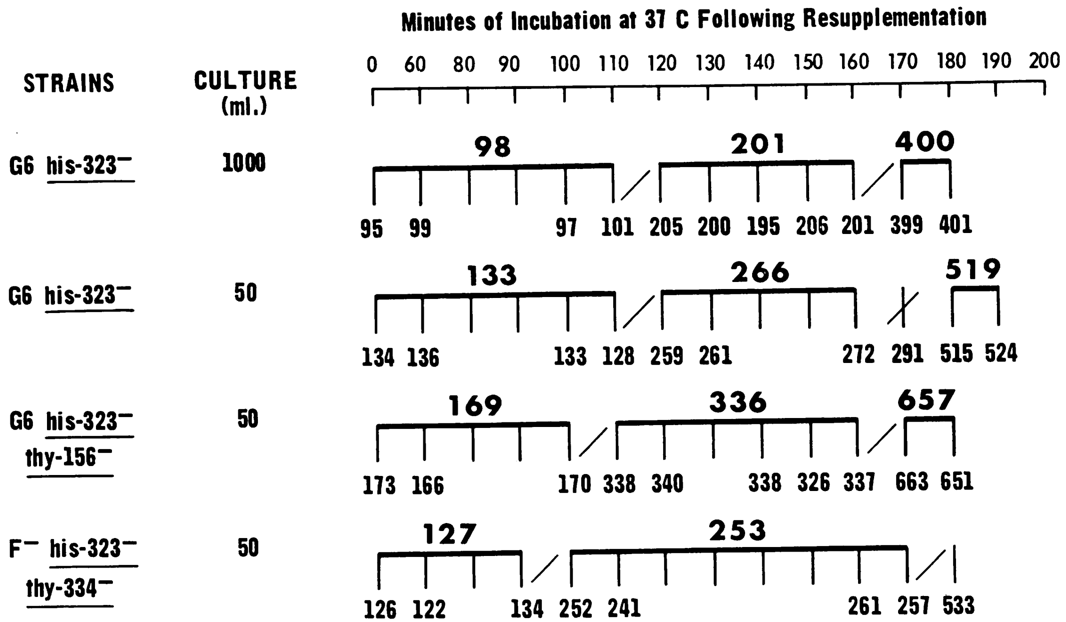
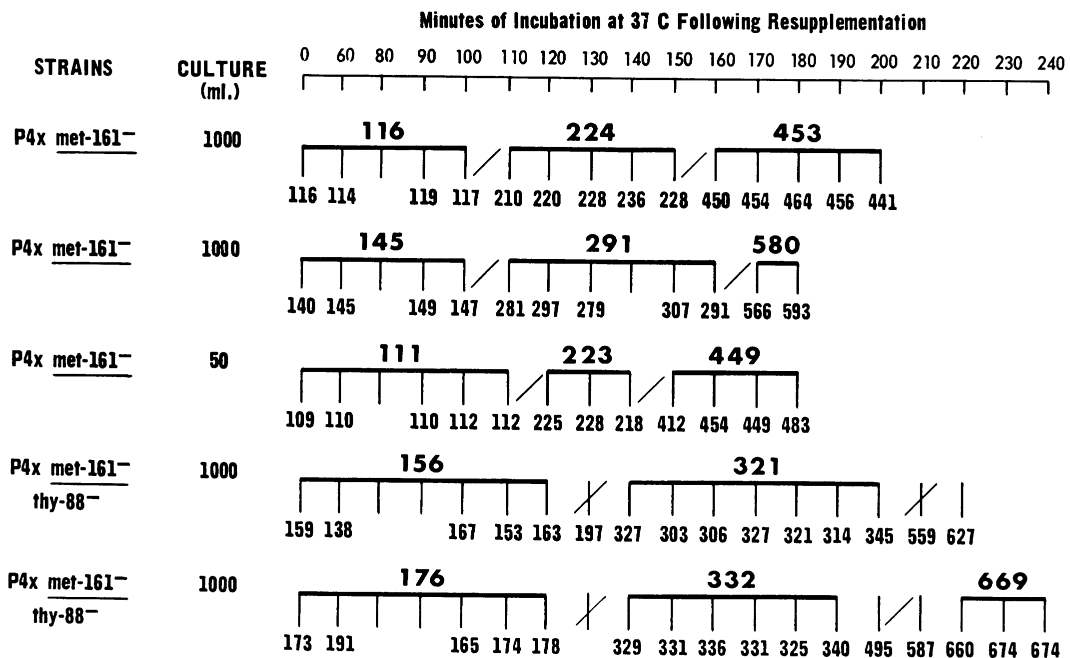


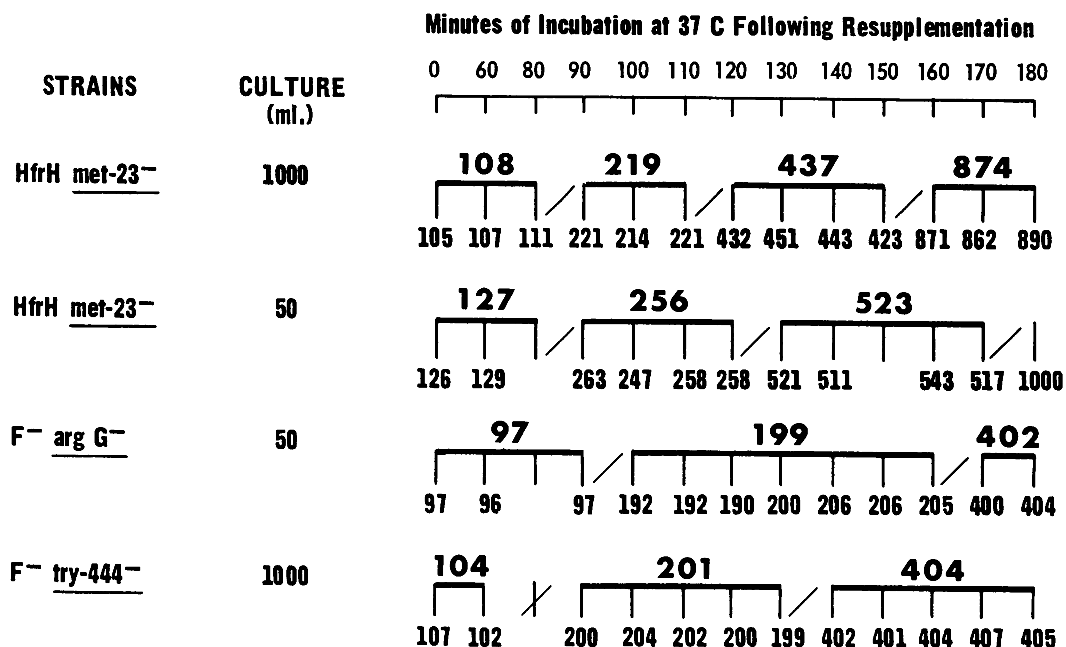
Table 3. PHASING OF DNA⁺·/RC^rel DERIVATIVES OF *E. coli* K12



the HfrH strain is shown in Fig. 3. In this case, the phased cycles were 35 min in duration, considerably shorter than the 53 min required for the OD to double in asynchronous growth (Table 1).

Additional phased growth data are compiled in Tables 2, 3, and 4. In these tables, the numbers below the small vertical (time) bars represent the total numbers of cells counted at the given time. The larger numbers above the dark hori-

Table 4. PHASING OF DNA^{+} /RCst DERIVATIVES OF E. coli K12



zontal bars represent the average plateau values. The slash mark indicates the time of division. Organisms included in Table 2 synthesize relatively small amounts of RNA and DNA during amino acid starvation. They are "wild type" for RNA synthesis, RC^{st}, and "mutant" with regard to their DNA-synthesizing capacity. The derivatives of *E. coli* K-12 included in Table 3 synthesize relatively large amounts of both RNA and DNA after withdrawal of a required amino acid; they are "mutant" for RNA, RC^{rel}, and "wild type" for DNA-synthesizing capacity. Cultures included in Table 4 are "wild type" for both types of nucleic acid synthesis. It may be noted that results for a given strain were generally reproducible, although the second and third phased divisions appeared slightly later in small-volume cultures.

The syntheses of protein, RNA, and DNA were followed during depletion of methionine by P4X (Fig. 4A) and HfrH (Fig. 4B), histidine by G6 (Fig. 4C), and tryptophan by 444 (Fig. 4D). In the depleting culture of G6, all three types of synthesis were arrested at the same time (Fig. 4C). The P4X derivative continued to synthesize both RNA and DNA for some time after protein synthesis was arrested (Fig. 4A). The HfrH and 444 derivatives arrested RNA and protein syntheses simultaneously, whereas DNA synthesis continued for some time (Fig. 4B and 4D). Thus, the synthesis of a nucleic acid past the point of protein-synthesis arrest during depletion paralleled the given strain's ability to synthesize that nucleic acid when its required amino acid was suddenly withdrawn (Table 1). It was somewhat surprising to find that, despite these major differences in synthetic capacities during amino acid depletion, the growth of each of these organisms after resupplementation demonstrated completely phased cell division (Tables 2, 3, and 4).

Discussion

All nutritionally deficient derivatives of *E. coli* K-12 tested have been phased with regard to cell division by the depletion method. The phenomenon seems independent of F (Hfr and F⁻ strains were employed), the amino acid requirement (mutants involving deficiencies in the biosynthesis of histidine, methionine, arginine, and tryptophan were used), the capacities to synthesize RNA and DNA during amino acid starvation, the generation time in supplemented minimal media (50 to 70 min), and thymine deficiencies. It was found that 1 μg/ml of the required amino acid was effective in the depletion phasing of all strains, despite the observation that a greater amount of histidine (100 μg/ml) than of the other requirements (20 μg/ml) was required to support optimal growth.

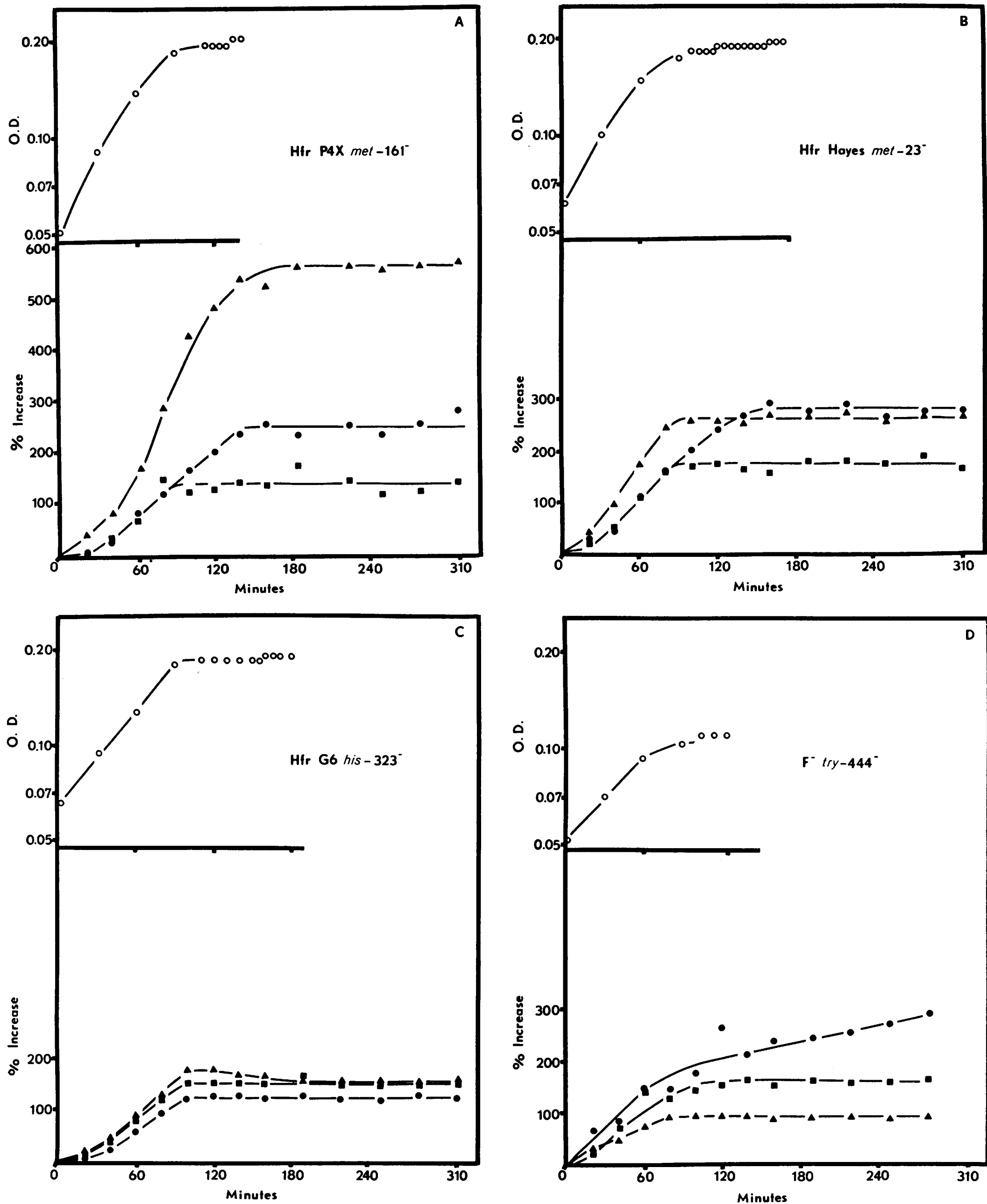


FIG. 4. *Increases in optical density* (○), *DNA* (●), *RNA* (▲), *and protein* (■) *by Escherichia coli K-12 cultures during depletion of 1 μg/ml of the required amino acid.*

It should be emphasized that the phasing of cell division following the growth depletion of a small amount of required amino acid contrasts with the lack of such phasing following the abrupt withdrawal of and starvation for a required amino acid (5).

In general, the generation time, i.e., the incubation time required for an asynchronous culture growing in supplemented minimal medium to double in OD, compared favorably with the time of the synchronized division cycle. The most notable exception was the HfrH culture that

displayed a 53-min doubling time and a 35-min cycle.

No difficulties were encountered when culture volumes were scaled up from 50 ml to 1 liter. For a given strain, both culture volumes followed the same growth characteristics during depletion and displayed comparable patterns of essentially completely phased division after resupplementation.

The early discovery that freshly depleted populations contained two portions, each with a separate division pattern emerging after resupplementation, suggested that the last division accomplished in the depleting culture provided each daughter cell with a distinct program for division. If such populations were starved for a sufficient time prior to resupplementation, then the two division schedules merged. A search for two distinct morphological types in freshly depleted cultures was unrewarding. A study has been initiated to determine at what point during the depletion growth the bacteria become committed to phased division following resupplementation.

Work is in progress to determine whether the DNA-synthesis cycle is geared to the division processes when cells are phased by the amino acid depletion method.

Acknowledgments

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