Metabolism of Thymineless Mutants of *Escherichia coli*

I. Absence of Thymidylate Synthetase Activity and Growth Characteristics of Two Sequential Thymineless Mutants

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A study of optimal thymine and deoxythymidine (dThd) growth requirements of the thymineless mutants of *Escherichia coli* 15, *E. coli* 70-462 (strain 70), and a variant, *E. coli* 70V3-462 (strain 70V3), showed that for maximal turbidity (growth) strain 70 required 10-fold greater concentrations of thymine or dThd than did strain 70V3. On suboptimal concentrations of thymine or dThd, growth of strain 70 was greater on dThd than on thymine. In contrast, maximal growth of strain 70V3 was the same on equimolar concentrations of thymine and dThd. Growth rate of strain 70V3 was the same on equimolar concentrations of thymine and dThd up to 4 μM ; at concentrations of 5 μM and greater, the "4-hr" growth was lower on dThd than on corresponding concentrations of thymine. Cultures of both thymineless mutants synthesized equal maximal amounts of DNA. Whereas strain 70V3 incorporated a maximum of 90% of the thymine or dThd in the media, strain 70 incorporated a maximum of only 10%. This poor utilization by strain 70 was neither a result of thymine or dThd conversion to a low-molecular-weight thymine derivative nor the production of a nonthymine inhibitory substance. Since strains 70 and 70V3 exhibited no thymidylate synthetase activity, the first mutation (strain 15 to strain 70) resulted in the loss of this activity. The second mutation (strain 70 to strain 70V3) probably brought about the loss of an enzyme(s) that catabolizes deoxyribose phosphate, permitting a greater net synthesis of dThd from thymine.

In 1947 Roepke and Mercer (23) reported the isolation of an X ray-induced thymine-requiring mutant of *Escherichia coli* strain 15 (ATCC 9723). This mutant, designated strain 70-462 (ATCC 9723h), spontaneously mutates to strains 70V-462 (22). (The original mutant, strain 70-462, will be referred to as strain 70; one of its variants, strain 70V3-462, as strain 70V3; and the parent wild strain, *E. coli* strain 15, as strain 15.) *E. coli* 15T⁻ (3) and strain I (25), two other thymineless strains, are derived from strain 70, as noted previously (6), and lack the enzyme thymidylate synthetase (4, 17). Experimental proof has not been presented that strain 70 or strain 70V3 lack thymidylate synthetase activity. Our intent was to identify the mutation in strain 70 and the additional mutation in strain 70V3. The results presented in this paper identify the mutation in strain 70 as loss of thymidylate synthetase and shed light on the other mutation in strain 70V3.

In a previous communication, we first noted

that strain 70 had a 10-fold greater thymine requirement than did strain 70V3 and, unlike strain 70V3, grew better on deoxythymidine (dThd) than on thymine (6). That the loss of thymidylate synthetase is the first mutation (strain 15 $\rightarrow$ strain 70) and not the second mutation (strain 70 $\rightarrow$ strain 70V; strain 15T⁻; strain I) was also indicated. Similar results have since been observed by Harrison (14) for other thymineless mutants derived from strain 15.

MATERIALS AND METHODS

E. coli strains 15, 70, and 70V3 were obtained from R. R. Roepke. Strains 15 and 70V3 were maintained on Nutrient Agar (Difco) slants. Strain 70 was maintained on Nutrient Agar slants supplemented with 200 μM thymine. Bacteria were grown overnight in minimal medium (10) containing 0.2% glucose (with 200 μM thymine for strain 70 and 20 μM thymine for strain 70V3). Cells were harvested by centrifugation, washed twice with minimal medium, resuspended at a concentration of approximately 2×10^8

cells per milliliter in minimal medium containing 0.2% glucose and thymine, and grown to approximately 6×10^8 cells per milliliter. These exponential-phase cells were washed as above, resuspended in minimal medium with 0.2% glucose, and transferred to Coleman round cuvettes (19 by 105 mm) containing minimal medium with 0.2% glucose and additives, as indicated, to give a final concentration of 3×10^7 cells per milliliter and a final volume of 10 ml. Incubation was at 37 C in a New Brunswick Gyrotory Shaker at maximal speed. Growth was followed turbidimetrically at 650 m μ in a Coleman Junior spectrophotometer. In exponentially growing cultures, the absorbancy varied linearly with cell number or cell mass up to 0.500, corresponding to 10^9 cells per milliliter or 0.5 mg (dry weight)/ml. Maximal growth is defined as the maximal absorbance (at 650 m μ) attained during the incubation period; "4-hr" growth is defined as the maximal absorbance attained at a time (usually 4 hr) when strain 70, growing on 100 μ M dThd, and strain 70V3, growing on 8 μ M dThd, reached an absorbancy of 0.450.

Thymidylate synthetase activity was assayed by method 8 described by Friedkin (12). Cell-free extracts were prepared by suspending 4×10^{10} exponential-phase cells in 10 ml of 0.05 M tris(hydroxymethyl)aminomethane (containing 0.01 M 2-mercaptoethanol and 0.001 M disodium ethylenediaminetetraacetic acid adjusted to pH 7.4 with HCl at 25 C) and sonically treating for 1 min with a Branson S-75 Sonifier (Branson Sonic Power, Danbury, Conn.) at 8 amp. During sonic treatment, the suspension was in a stainless-steel tube around which circulated coolant at -10 C. The suspension was centrifuged at $30,000 \times g$ for 15 min; the supernatant fluid, containing approximately 1 mg of protein per ml, was used for the thymidylate synthetase assay.

Radioactive thymine and dThd incorporation into deoxyribonucleic acid (DNA) was measured under conditions employed for the bacterial growth studies, except that thymine-2- 14 C, 30 mc/mmole, and dThd-2- 14 C, 30 mc/mmole (purchased from New England Nuclear Corp., Boston, Mass.) were added; 1-ml samples were removed at time intervals, cooled in an ice bath, and adjusted to a final concentration of 5% trichloroacetic acid. After at least 15 min, the precipitates were collected on Millipore or Gelman membrane filters (0.45- μ pore size) and washed with cold 5% trichloroacetic acid. The filters were placed in liquid scintillation counting vials and dried in an oven at 55 C. Toluene phosphor solution (5 ml) was added; radioactivity was determined in a liquid scintillation spectrometer. The counting efficiency was approximately 80% from 3×10^7 to 2×10^9 cells per filter.

The distribution of radioactivity in the media was determined on the cultures employed for measuring 14 C-thymine and 14 C-dThd incorporation into DNA. The cultures were filtered; portions of the filtrates were applied to Whatman 3 MM filter paper and chromatographed (descending) in solvent 8 (11). The paper was cut into segments (1.3 cm in the direction of solvent flow and 2.5 cm wide), placed in liquid scintillation vials, and counted in a liquid

scintillation spectrometer after addition of toluene phosphor. The counting efficiency was approximately 65%.

RESULTS

Thymine and dThd growth requirements. Figure 1 shows the maximal growth-concentration curves of strain 70 and strain 70V3 on thymine and dThd. The thymine or dThd requirement for maximal growth was greater for strain 70 than for strain 70V3. The optimal concentrations were 100 μ M thymine or dThd for strain 70 and 8 μ M thymine or dThd for strain 70V3. At suboptimal concentrations of dThd and thymine, the growth attained by strain 70 was greater on dThd than on thymine. In contrast, strain 70V3 responded equally on equimolar concentrations of thymine or dThd.

Although the final growth of strain 70 was the same on 100 μ M thymine or dThd, growth at earlier time periods was greater on 100 μ M dThd than on 100 μ M thymine (Fig. 2). This was also true on other equimolar concentrations of thymine and dThd. Growth of strain 70 on low concentrations of thymine or dThd was characterized by breaks in the time-turbidity curves. For ex-

FIG. 1. Growth of strains 70 and 70V3 in minimal medium containing 0.2% glucose and various concentrations of thymine (O) and dThd (▲).

FIG. 2. Differences in the time-turbidity curves of strain 70 on various concentrations of thymine and dThd.

ample, on 40 μM thymine, a marked change occurred in the growth rate at 1.5 and at 3 hr. In contrast to strain 70, the time-turbidity curves of strain 70V3 on thymine or dThd exhibited no marked change in growth rate preceding maximal turbidity (Fig. 3).

The difference in growth response of strains 70 and 70V3 on dThd and thymine (Fig. 2 and 3) is accentuated by plotting "4-hr" growth. The "4-hr" growth-concentration curves for strain 70 (Fig. 4) demonstrate that the thymine or dThd requirement for optimal "4-hr" growth (200 μM) is greater than that obtained from the maximal growth concentration curves in Fig. 1 (100 μM). This greater thymine and dThd requirement is reflected throughout the "4-hr" growth curves. Growth of strain 70V3 was optimal on 8 μM thymine from either the maximal or "4-hr" growth curves (Fig. 1 and 4). Although the maximal growth of strain 70V3 was equal on dThd and thymine (Fig. 1), the "4-hr" growth on dThd concentrations of 5 μM and greater was inhibited relative to growth on thymine (Fig. 4). This inhibition was expressed primarily as a longer lag phase on concentrations of dThd above approximately 20 μM (Fig. 3). High concentrations of thymine had no similar effects. Concentrations of

dThd as high as 2 mM were without effect on growth of strains 70 or 15.

From the data for strain 70 in Fig. 1 and 4, the thymine and dThd concentrations that yielded equal growth were plotted (Fig. 5). The initial slope of the curve (up to 110 μM dThd) obtained from the "4-hr" growth data has a value of 1.45 μmoles of thymine per μmole of dThd or, from the reciprocal, 0.7 μmole of dThd per μmole of thymine.

Extent of incorporation of thymine and dThd. Table 1 shows values for maximal incorporation of various concentrations of ^{14}C -labeled thymine and dThd into DNA of strains 15, 70, and 70V3. The greatest incorporation for both strains 70 and 70V3 was approximately the same (18 μmoles of thymine or dThd per ml of culture). The greater growth attained in this experiment, as compared to the maximal growth shown in Fig. 1, is ascribed to greater aeration resulting from removal of samples during the incubation. In agreement with Barner and Cohen (3), these experiments emphasize the inadequacy of turbidimetric measurements for determining thymine and dThd requirements of the thymineless strains. Qualitatively, however, the radioactive incorporation and turbidimetric data agree in showing

FIG. 3. Differences in the time-turbidity curves of strain 70V3 on various concentrations of thymine and dThd.

larger thymine and dThd requirements of strain 70 as compared to strain 70V3, greater growth of strain 70 on dThd than on thymine, and equivalent maximal growth of strain 70V3 on thymine or dThd. In addition, the maximal percentage of the initial concentrations of thymine or dThd incorporated can be calculated. At concentrations of thymine of 100 to 150 μM , and of dThd of 40 to 150 μM , strain 70 incorporated 10%; at thymine or dThd concentrations of 3.3 to 10.3 μM , strain 70V3 incorporated 90%.

Fate of thymine and dThd. The results in Table 1 indicated that strain 70 required a concentration of thymine or dThd that was approximately 10-fold greater than the amount utilized for DNA-thymine synthesis. This raised a question regarding the fate of the excess thymine and dThd. When either thymine or dThd was initially present in the medium, approximately 95% of the radioactivity in media in which strains 70, 70V3, and 15 (data not shown) had synthesized maximal amounts of DNA was found in thymine (Fig. 6). Thus, the very high thymine and dThd requirements of strain 70 were not a result of thymine or dThd conversion to any known low-molecular-weight thymine derivative (e.g., ribosylthymine) which is not utilized efficiently for DNA-thymine synthesis. Similar results were obtained by spectrophotometric analysis of the

FIG. 4. "Four-hour" growth of strains 70 and 70V3 in minimal medium containing 0.2% glucose and various concentrations of thymine (O) and dThd (Δ).

FIG. 5. Comparison of the thymine and dThd concentrations that yielded equal growth of strain 70, from maximal growth (O, data from Fig. 1) and "4-hr" growth (Δ, data from Fig. 4). The equivalence curve (broken line) would have been obtained if growth were equal on thymine or dThd.

spent media for thymine (16) or by determining thymine concentration microbiologically with strain 70 or strain 70V3 as the test organism. These latter experiments also indicated that strain 70 did not excrete any nonthymine inhibitory substances.

Thymidylate synthetase activity. Barner and Cohen (4), using *E. coli* 15T⁻, and Mantavinos and Zamenhof (17), using strain I, have shown that these two mutants of strain 70 have no thymidylate synthetase activity. Thymidylate synthetase activity was also absent in strains 70 and 70V3 (Table 2). The absence of thymidylate synthetase activity in strain 70 probably was not due to the presence of an inhibitor, since addition of a sonic extract of strain 70 had no effect on the thymidylate synthetase activity of strain 15 (Table 2). These results indicate that the loss of thymidylate synthetase activity resulted from the mutation of strain 15 to strain 70 and not the mutation of strain 70 to strain 70V3.

DISCUSSION

The most marked phenotypic difference between strains 70 and 70V3, two thymineless strains of *E. coli* 15, was the higher-thymine or dThd growth requirements of strain 70. Inability of wild-type *E. coli* cells to incorporate exogenous thymine into DNA and their resistance to growth inhibition by thymine analogues has been re-

TABLE 1. Incorporation of various concentrations of ^{14}C -labeled thymine and deoxythymidine (dThd) into DNA of *Escherichia coli* strains, 15, 70, and 70V3

Strain	Thymine				dThd			
	Concn (μM)	A^a	Amt incorporated ($\mu\text{moles/ml}$)	Per cent of total incorporated/ml	Concn (μM)	A^a	Amt incorporated ($\mu\text{moles/ml}$)	Per cent of total incorporated/ml
15	16.7 (0.5) ^b	0.650	0.062	0.37	16.7 (0.5)	0.650	0.25	1.5
70	41 (0.5)	0.155	1.9	4.6	41 (0.5)	0.290	4.0	9.8
	101 (0.5)	0.640	10.0	9.9	101 (0.5)	0.645	10.6	10.5
	121 (0.5)	0.665	12.2	10.1	121 (0.5)	0.645	13.8	11.4
	151 (0.5)	0.650	14.6	9.7	151 (0.5)	0.645	15.3	10.1
	201 (0.5)	0.655	14.0	7.0	201 (0.5)	0.645	16.0	8.0
	1,000 (0.5)	0.650	17.3	1.73	1,000 (0.5)	0.635	20.0	2.0
70V3	3.3 (0.1)	0.390	2.8	85	3.3 (0.1)	0.380	3.0	91
	5.3 (0.1)	0.530	4.7	89	5.3 (0.1)	0.540	4.6	87
	8.3 (0.1)	0.640	7.2	87	8.3 (0.1)	0.640	7.2	87
	10.3 (0.1)	0.650	9.0	87	10.3 (0.1)	0.645	9.1	88
	50 (0.3)	0.650	17.7	35	50 (0.3)	0.650	17.6	35

^a Absorbance at 650 $\text{m}\mu$.^b Numbers in parentheses refer to the microcuries (per milliliter) of thymine-2- ^{14}C or dThd-2- ^{14}C .

ported (7, 9, 24). Compared to thymine, dThd is efficiently incorporated into the DNA of a variety of bacteria (2, 15, 20). However, even on high concentrations of dThd and relatively low concentrations of cells, the efficient incorporation of dThd proceeds for a relatively short time because of its rapid conversion to thymine by thymidine phosphorylase, an enzyme induced by dThd and other deoxynucleosides (6, 20, 21). Thus, the first mutation, loss of thymidylate synthetase (Table 2), yielded a mutant (strain 70) which, like strain 15, utilizes thymine inefficiently. Because of a second mutation, strain 70V3 utilizes thymine and dThd efficiently and equally for DNA synthesis (Table 1).

Differences appear in published reports on the growth response to thymine and dThd of several variants of strain 70. Roepke (*personal communication*) with strain 70V1 and Zamenhof and Griboff (25) with strain I reported better growth on dThd than on thymine; Barner and Cohen (3) with strain 15T⁻ found equal growth on equimolar concentrations of thymine and dThd. These differences in growth response of variants of strain 70 to thymine and dThd are probably a result of experimental conditions, although differences in mutation site of the variant strains cannot be excluded. Both Roepke and Zamenhof and Griboff (25) employed small inocula (approximately 10^4 to 10^5 cells per milliliter) previously grown on enriched medium, and their incubations were conducted without shaking. Barner and Cohen (3) and we employed larger

inocula (10^7 cells per milliliter) previously grown in minimal medium, and our incubations were conducted with vigorous aeration. Under our conditions, strains 70V3, 15T⁻, and I exhibited identical growth curves on various thymine and dThd concentrations.

Roepke (22) presented evidence that adaptation of cultures of strain 70 grown on limiting thymine is a result of mutation and not enzymatic adaptation. This mutation was not identified by Roepke nor by others who have worked with similar or identical mutants. Results presented in this paper show that strains 15, 70, and 70V3 neither convert thymine or dThd to an inhibitory or nonutilizable form (Fig. 6) nor produce a non-thymine inhibitory substance. Another possible mutation, the ability of strain 70V3 to transport thymine or dThd from the media into the cell at a much greater rate than does strain 70, has also been excluded (Breitman and Bradford, *in preparation*).

Greater thymine and dThd requirements of strain 70 as compared to strain 70V3, and greater growth response of strain 70 on dThd than on thymine, indicate that the mutation to strain 70V3 permits a greater net synthesis of dThd. On low levels of thymine, the net rate of synthesis of dThd by strain 70 is not great enough to meet the requirements for DNA synthesis. Low levels of dThd can support the DNA-thymine requirement for only a relatively short time because of rapid degradation to the poorly utilized thymine by thymidine phosphorylase. This difference in

growth response of strain 70 to thymine and dThd mirrors the observations discussed above on incorporation of dThd but not thymine into wild-type *E. coli* DNA.

Because strain 70 can incorporate thymine into DNA, it appears to have gained a metabolic pathway that strain 15 lacks. An explanation is that the pool of endogenous deoxyribonucleoside in strain 15 is too small to support thymine-to-dThd conversion at a rate commensurate with the rate of DNA synthesis. This view is strengthened by the observation of Boyce and Setlow (5) that addition of deoxyadenosine markedly promotes incorporation of thymine or dThd into wild-type *E. coli* DNA

TABLE 2. *Thymidylate synthetase activity of Escherichia coli* strains 15, 70, and 70V3

Strain	Protein ^a	dTMP formed ^b
15	0.53	31.9
	1.05	60.0
70	0.96	<1
70V3	0.88	<1
15 + 70	0.53 + 0.48	30.0

^a Expressed as milligrams of protein per assay.

^b Expressed as millimicromoles of deoxythymidine monophosphate per hour per assay.

FIG. 6. Distribution of radioactivity in medium after maximal growth of strains 70 and 70V3 on ¹⁴C-thymine and ¹⁴C-dThd. Filtrates, from cultures employed to obtain data in Table 1, were chromatographed; total radioactivity applied was approximately 15,000 counts/min for strain 70 and approximately 9,000 counts/min for strain 70V3. The relative mobilities were: thymine, 1.0; dThd, 0.64; ribosylthymine, 0.36. Parenthetical numbers are the percentages of the total radioactivity applied that were recovered in the thymine area. Radioactivity between 0 and 6 inches (15 cm) on chromatograms is from radioactive contaminants in the commercial ¹⁴C-dThd preparations.

For strains 70 and 70V3, a major source of deoxyribose for thymine-to-dThd conversion could be deoxyuridine formed by dephosphorylation of deoxyuridylate, accumulating because of the absence of thymidylate synthetase activity. In strain 70, deoxyuridine or dThd would be degraded rapidly by thymidine phosphorylase if high concentrations of thymine, a potent inhibitor of this enzyme, were not present (13, 21). A similar degradation would also be expected in strain 70V3, but our previous finding (6), that strain 70V3 and not strain 70 excretes large quantities of deoxyribose into the medium during thymine starvation, indicates that strain 70V3 has lost one of the enzymes involved in the degradation of deoxyribose. (Recent results indicate that deoxyribose 5-phosphate aldolase activity is absent or present at a very low level in sonic extracts of strain 70V3 as compared to sonic extracts of strains 70 and 15.) Inability to catabolize deoxyribose may result in a decreased net catabolism of dThd as well as of the other deoxynucleoside(s) involved in thymine-to-dThd conversion. Apparently, the high thymine concentration required for optimal growth of strain 70 and the mutation in strain 70V3, though acting by different mechanisms, result in a greater net synthesis of dThd.

Harrison (14) suggested that the high thymine requirement of a strain 70-type thymineless mutant, designated strain 15-1, was due to an endogenous inhibitor. His finding that growth of strain 15-1 on thymine was inhibited by exogenous cytidine and uridine and enhanced by exogenous deoxycytidine or deoxyuridine was interpreted as being consistent with an increased level of endogenous inhibitor in the presence of cytidine or uridine and a decreased level or an overcoming of the inhibitor by deoxycytidine or deoxyuridine or by high concentrations of thymine. However, no experimental evidence for the presence of an endogenous inhibitor was presented.

The inhibitory effects of cytidine and uridine reported by Harrison and previously found in strain 15T⁻ (7) and strain I (26) can be explained by the effect of these compounds on conversion of thymine to ribothymidine, a compound poorly utilized by strain 15T⁻ and whose addition to media containing thymine inhibits growth (7). Mantsavinos and Zamenhof (17) have shown that conversion of thymine to ribothymidine by cell-free extracts of strain 15 is markedly increased by the addition of adenosine. Although other ribonucleosides were not tested, it can be assumed that cytidine or uridine would also enhance this reaction.

An explanation for the growth-enhancing effects of deoxycytidine and deoxyuridine described by Harrison is that these compounds promote the conversion of thymine to dThd, as has been shown in cell-free extracts (17) and was inferred from experiments with growing cells (5). This explanation is consistent with our proposition, described more fully above, that in strain 70 the endogenous deoxyribonucleoside pool is too small to support adequate thymine-to-dThd conversion on low thymine concentrations.

The existence of strain 70- and strain 70V3-type mutants probably is not unique for strain 15. Alikhanian et al. (1), using the aminopterin technique of Okada et al. (19), isolated thymineless mutants of *E. coli* K-12 lacking thymidylate synthetase. Some of these mutants were reported to mutate to strains having 10-fold lower thymine requirements. Although these workers have called the latter mutants revertants, this study and those of Roepke (22) and Harrison (14) indicate that these mutants and other commonly employed thymineless strains are likely double mutants of the strain 70V type.

The mechanism for the inhibitory effect of dThd on growth of strain 70V3 must await further experimentation. In mammalian systems, inhibition by dThd (8, 18) has been shown to be relieved by deoxycytidine, implicating an inhibition of the enzyme ribonucleotide reductase by deoxythymidine triphosphate. Attempts to relieve the dThd inhibitory effects on strain 70V3 by addition of deoxycytidine have not been successful.

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