JOURNAL OF BACTERIOLOGY, Nov. 1967, p. 1546-1550
Vol. 94, No. 5
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Thymidine and Thymine Incorporation into Deoxyribonucleic Acid: Inhibition and Repression by Uridine of Thymidine Phosphorylase of *Escherichia coli*

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Received for publication 27 July 1967

Thymidine is poorly incorporated into deoxyribonucleic acid (DNA) of *Escherichia coli*. Its incorporation is greatly increased by uridine, which acts in two ways. Primarily, uridine competitively inhibits thymidine phosphorylase (E.C.2.4.4), and thereby prevents the degradation of thymidine to thymine which is not incorporated into normally growing *E. coli*. Uridine also inhibits induction of the enzyme by thymidine. It prevents the actual inducer, probably a deoxyribose phosphate, from being formed rather than competing for a site on the repressor. The inhibition of thymidine phosphorylase by uridine also accounts for inhibition by uracil compounds of thymine incorporation into thymine-requiring mutants. Deoxyadenosine also increases the incorporation of thymidine, by competitively inhibiting thymidine phosphorylase. Deoxyadenosine induces the enzyme, in contrast to uridine. But this is offset by a transfer of deoxyribose from deoxyadenosine to thymine. Thus, deoxyadenosine permits incorporation of thymine into DNA, even in cells induced for thymidine phosphorylase. This incorporation of thymine in the presence of deoxyadenosine did not occur in a thymidine phosphorylase-negative mutant; thus, the utilization of thymine seems to proceed by way of thymidine phosphorylase, followed by thymidine kinase. These results are consistent with the data of others in suggesting that wild-type *E. coli* cells fail to utilize thymine because they lack a pool of deoxyribose phosphates, the latter being necessary for conversion of thymine to thymidine by thymidine phosphorylase.

Thymidine (TdR) incorporation into deoxyribonucleic acid (DNA) of microoorganisms stops after only a short time, owing to degradation of TdR to thymine (1, 17) by an inducible TdR phosphorylase (E.C.2.4.2.4) (12). The bacteria are unable to incorporate the thymine that is produced (6). Thus, mutants that lack TdR phosphorylase effectively incorporate TdR because they do not degrade it (7). Addition of deoxyadenosine (AdR) permits extensive incorporation of TdR into wild-type *Escherichia coli* (2, 10), since AdR can transfer deoxyribose to thymine and reform TdR.

But uridine and other ribosides enhance TdR incorporation (2), although they cannot provide deoxyribose. As possible explanations, uridine might inhibit the activity of TdR phosphorylase or it might repress induction of the enzyme. Experiments presented here suggest that both of these mechanisms can be important in enhancing TdR incorporation.

MATERIALS AND METHODS

E. coli B was taken from the laboratory stock. *E. coli* K-12-SH and the TdR phosphorylase-negative mutant K-12-SH-28-15A were obtained from W. L. Fangman. The bacteria were grown on minimal medium M63, plus 5 μg/of thiamine per ml with 2 mg of glycerol per ml as the carbon source. Incubation was at 37 C with aeration by swirling (17). Inducers and other compounds were added to the culture in exponential growth when the cell concentration was about 3×10^8/ml, as measured by turbidity with a Klett colorimeter. Samples for assay were taken at times indicated in the text.

To determine TdR phosphorylase activity, the bacteria were separated by centrifugation, washed in 0.1 M tris(hydroxymethyl)aminomethane buffer (*p*H 7.2) and resuspended in one-fifteenth the original volume of 0.1 M phosphate buffer (*p*H 5.9). They were

disrupted with a Branson Sonifier at position 4 for 1.5 min and then centrifuged; 0.5 mg of dry dihydrostreptomycin sulfate was added per ml of supernatant fluid to precipitate nucleic acids. After centrifugation, the supernatant fluid was used for enzyme assays. In a total volume of 1 ml of buffer (*p*H 5.9), 0.04 ml of extract was added, and, after incubation at 37 C with TdR, the thymine produced was determined by the increase in optical density at 300 mμ (*p*H 12) above that of a control lacking TdR (17).

^{3}H-TdR (specific activity, 8.9 c/mmole) was obtained from Nuclear Research Chemicals, Inc., Orlando, Fla., and from Schwarz Bio Research, Inc., Orangeburg, N.Y.

^{3}H-thymine (specific activity, 3.2 c/mmole) was obtained from New England Nuclear Corp., Boston, Mass. Nonradioactive TdR and AdR were obtained from Sigma Chemical Co., St. Louis, Mo., and uridine, from Schwartz Bioresearch. To measure the uptake of radioactive TdR (or thymine) into intact bacteria, 1 μg of ^{3}H-TdR (or thymine) per ml, plus other compounds as indicated in the text was added to 25 ml of exponentially growing *E. coli* (2 $\times$ 10^8/ml). Growth was continued at 37 C, and at 30-min intervals 2-ml samples were withdrawn, filtered on HA filters (Millipore Corp., Bedford, Mass.), and washed with 20 ml of 0.1 M sodium phosphate buffer (*p*H 7.0) at 0 C. The filters were counted in a Packard liquid scintillation counter, to give total ^{3}H uptake by the bacteria. To determine incorporation into trichloroacetic acid-precipitable material, 2-ml samples were placed into test tubes containing 2 ml of 15% trichloroacetic acid and 0.8 mg of TdR. They were filtered as above, washed with 10 ml of 10% trichloroacetic acid then with 15 ml of 50% ethyl alcohol, and counted.

RESULTS

Inhibition of TdR phosphorylase activity. Neither AdR nor uridine is a substrate of TdR phosphorylase (19). Both showed competitive inhibition-type kinetics at a concentration higher than their apparent K_i values (1.1 and 0.7 mM, respectively) and at TdR concentrations greater than K_m, which was 1.3 mM (Fig. 1). K_m is in reasonable agreement with a previously determined value of 1.1 mM (17). Although inhibition by uridine is probably competitive, the effect of AdR might be more complex owing to production of deoxyribose-1-phosphate from it (13), thus permitting reutilization of thymine.

Effects of induction. Cultures were exposed to nucleosides for 1 or 2 hr to permit extensive induction (17). Experiments 1 and 2 (Table 1) were done with the relatively high concentrations required for induction (17, 18). Both TdR and AdR were good inducers, but uridine did not induce. It inhibited induction by TdR about 75% but had relatively little effect on induction by AdR. The third experiment shows that the concentration

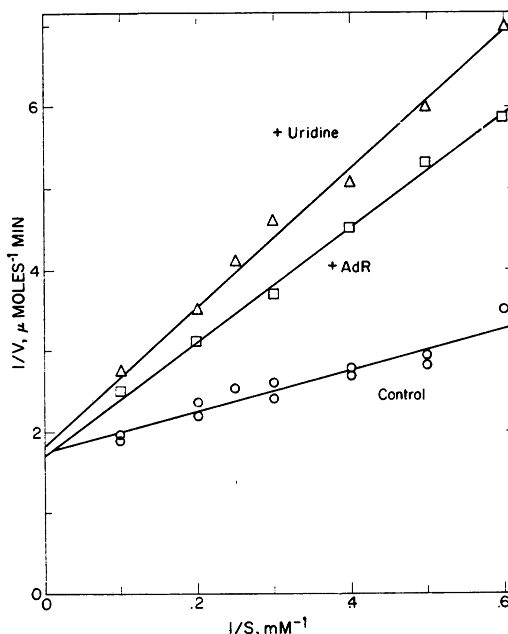

FIG. 1. *Inhibition of thymidine phosphorylase activity by uridine or AdR. Thymine production in 40 sec by an extract made from induced bacteria (growth with 2 mM thymidine to resting state) was determined as described in Materials and Methods, with various concentrations of TdR and with or without 1.9 mM AdR or 2.05 mM uridine. Units of velocity are micromoles of thymine produced per minute by extract from approximately 10^9 Escherichia coli B.*

of TdR used for incorporation studies did not induce the enzyme.

Uptake and incorporation of TdR. AdR and also various ribonucleosides permit effective incorporation of TdR into DNA of *E. coli* (2). Even as little as 1 μg/ml of TdR was incorporated into cold trichloroacetic acid-insoluble material for 2 hr when either AdR or uridine was present, as compared to minor incorporation with TdR-alone (Fig. 2). It is surprising that the AdR was so long-lasting, since other data suggest that it should all have been degraded within 0.5 hr (13). Approximately 10% of the added TdR was incorporated. This amounts to 4% of the total thymine of the newly formed DNA, calculated from the known DNA content of *E. coli*. Therefore, most of this thymine must have been synthesized by the de novo pathway.

Uptake of radioactivity into the intact bacteria is also shown in Fig. 2. The difference of each pair of curves is the pool within the cells. Either AdR or uridine permitted a larger intracellular pool or radioactive material to be formed from TdR than was obtained with TdR alone.

TABLE 1. *Induction of thymidine phosphorylase*[a]

Expt	Induction time	Present during induction			Assay TdR	Activity
		TdR	AdR	Uridine		
	hr	mM	mM	mM	mM	
1	1.0	2.0	—	—	2.0	.20
		2.0	5.0	—	2.0	.15
		2.0	—	5.0	2.0	.05
2	1.0	—	—	—	0.83	.005
		—	1.0	—	0.83	.060
		—	—	2.0	0.83	.007
		—	1.0	2.0	0.83	.050
3	2.0	—	—	—	1.05	.001
		0.004	—	—	1.05	.004
		0.004	1.5	—	1.05	.15
		0.004	—	1.5	1.05	.003

[a] *Escherichia coli* B in exponential phase was grown for the indicated time with the compounds listed as "Present during induction." Extracts made as described in Materials and Methods were assayed for thymidine phosphorylase. Enzyme activity is given as micromoles of thymine produced per minute by extract from approximately 10^9 bacteria.

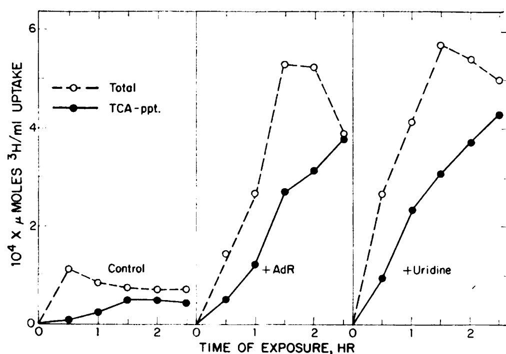

FIG. 2. *Uptake of ^{3}H-TdR into intact Escherichia coli B and trichloroacetic acid-insoluble material. The uptake from 1 μg/ml of TdR (0.004 mM) in the absence or presence of 1.5 mM AdR or uridine was determined as described in Materials and Methods.*

To judge the relative importance, for incorporation, of inhibition of enzyme activity and inhibition of induction by uridine, the ability of preinduced bacteria to incorporate TdR was determined. In experiments like those shown in Fig. 2, after 1 hr of growth in the presence of 1 mM TdR (to induce TdR phosphorylase) the bacteria were washed and resuspended in fresh medium; ^{3}H-TdR incorporation into trichloroacetic acid-insoluble material was then determined. With TdR alone, only 0.5% of the radioactivity was incorporated after 2 hr. When 1.5 mM AdR was present, 2% incorporation was obtained. This level of incorporation was found also when 1.5 mM uridine was present. Thus, AdR or uridine increased incorporation of TdR into induced bacteria, but not nearly as effectively as into uninduced bacteria (which incorporated about 10% of the added TdR). Therefore, inhibition of induction should enhance incorporation, and should supplement inhibition of the enzyme activity in increasing incorporation.

Thymine incorporation. The incorporation of TdR is aided by AdR, which can provide deoxyribose (13) to the thymine produced by action of TdR phosphorylase (14), thereby reforming TdR (2, 10). By the same mechanism, AdR should permit the incorporation of added ^{3}H-thymine into trichloroacetic acid-insoluble material. Incorporation in 1.5 hr reached 4% of the 1 μg/ml added (Fig. 3). Thymine incorporation was very small when AdR was absent, or when uridine was supplied, as would be expected, since uridine cannot supply deoxyribose. Thus, AdR permitted thymine incorporation not far inferior to TdR incorporation.

These results were obtained with TdR phos-

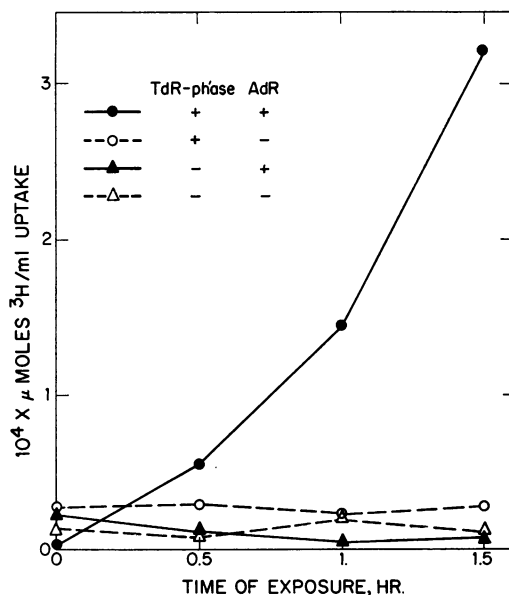

FIG. 3. *Incorporation of ^{3}H-thymine into trichloroacetic acid-insoluble material of thymidine phosphorylase-positive or -negative Escherichia coli K-12. The experiment was performed in the same way as in Fig. 2, except that 1 μg/ml of thymine (0.008 mM) with or without 1.5 mM AdR was used. Incorporation of radioactivity into trichloroacetic acid-insoluble material is plotted versus sampling time.*

phorylase-positive bacteria. When a thymidine phosphorylase-negative mutant was used, AdR did not permit the incorporation of thymine (Fig. 3). Therefore, the transfer of deoxyribose appears to require TdR phosphorylase.

DISCUSSION

Effects of AdR or uridine on TdR uptake are summarized in Table 2. AdR aids TdR incorporation by competitively inhibiting TdR phosphorylase, so as to prevent degradation of TdR, and also by reforming TdR from thymine by deoxyribose transfer (2, 10). The two effects might function sequentially as the enzyme is induced. Uridine probably principally inhibits TdR phosphorylase activity, since the enzyme is not induced by low concentrations of TdR. At high concentrations of thymidine, uridine probably also helps by blocking induction of the enzyme. Uridine cannot serve as a deoxyribose donor, nor is it split by thymidine phosphorylase (19).

The action of uridine as an "anti-inducer" is unusual; few cases of this sort have been reported (11, 15). Induction might be directly inhibited by competition of uridine with TdR for the macromolecular repressor. Alternatively, uridine could indirectly block induction, for example, by preventing conversion of TdR to the actual inducer, which is thought to be a deoxyribose phosphate or a related compound (14, 18). If the former hypothesis is correct, uridine should also inhibit induction by AdR, as it should compete with AdR for the binding site on the repressor. Since this inhibition was weak (Table 1), the latter mechanism is favored. AdR is converted to the inducer by a different pathway than is TdR, and presumably this conversion is not inhibited by uridine.

Mutants (thymidylate synthetase - negative) which have lost ability to synthesize thymidylate de novo simultaneously gain ability to utilize thymine, in contrast to their parental strains. Several suggestions have been made to explain this long-puzzling, apparently pleotropic mutation (6, 14). It now seems likely that a sufficient pool of deoxyribose phosphates is not available in the wild-type bacteria to permit thymine to be converted to TdR (3). But thymine-requiring mutants accumulate these necessary deoxynucleoside phosphates (16, 4) when the thymidylic acid supply limits their DNA synthesis. The observation presented here that TdR phosphorylase-negative mutants are unable to utilize thymine in the presence of a deoxyribose donor (AdR) supports this mechanism and indicates that the first step in thymine utilization is its conversion to TdR, catalyzed by TdR phosphorylase (14, 20).

The often made observation that uracil or uridine inhibits incorporation of thymine into thymine-requiring mutants (5, 8–10) is also explained, because uracil compounds inhibit TdR phosphorylase so that the enzyme cannot convert thymine to TdR.

ACKNOWLEDGMENTS

We are indebted to W. L. Fangman for the thymidine phosphorylase-negative strain and its parental type.

This investigation was supported by Public Health Service grant AI-04409 from the National Institute of Allergy and Infectious Diseases.

ADDENDUM IN PROOF

That the pathway of thymine incorporation is via TdR and dTMP is also demonstrated by the inability of a deoxythymidine kinase-deficient mutant to incorporate 2-^{14}C-TDR into DNA, either directly or after its conversion to 2-^{14}C-thymine by the thymidine phosphorylase present (9*a*).

TABLE 2. *Effects of AdR and uridine on thymidine incorporation*

Determination	Addition		
	None	AdR	Uridine
Thymidine incorporation	Poor	Prolonged	Prolonged
Intracellular pool	Low	Increased	Increased
Thymidine phosphorylase activity	Normal; K_m = 1.3 mM	Inhibited; K_I = 1.1 mM	Inhibited; K_I = 0.7 mM
Enzyme production	Low; induced by thymidine	Induced	Low; induction by thymidine inhibited
Thymine incorporation			
by thymidine phosphorylase$^+$	Negligible	Stimulated	Negligible
by thymidine phosphorylase$^-$	Negligible	Negligible	—
by thymidylate synthetase$^-$	High	—	Inhibited

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