Trimethoprim Action and Its Analogy with Thymine Starvation

S. G. B. AMYES AND J. T. SMITH

Department of Pharmaceutics, The School of Pharmacy, University of London, Brunswick Square, London, WC1N 1AX, United Kingdom

Received for publication 24 October 1973

In a minimal medium, trimethoprim is merely bacteriostatic on the prototroph *Escherichia coli* 114. The drug was bactericidal when the amino acids methionine and glycine, plus a purine or purine nucleoside, were also present. This response could be reversed completely when thymine and lysine were added to the culture. Methionine, glycine, and the purine are thought to maintain the integrity of the tetrahydrofolate pool under trimethoprim treatment and prevent the thymidylate synthetase reaction. Thus, the organism behaves phenotypically as a thymineless mutant. The mechanisms by which thymine and lysine reverse the bactericidal effect of trimethoprim in a minimal medium containing methionine, glycine, and adenine is discussed.

The enzyme dihydrofolate reductase (5, 6, 7, 8-tetrahydrofolate: nicotinamide adenine dinucleotide phosphate oxidoreductase, EC 1.5.1.3) reduces dihydrofolic acid (DHF) to tetrahydrofolic acid (THF). The bacterial enzyme can be selectively inhibited by the antifolate drug trimethoprim (Tm) (20), which is virtually without effect on the mammalian enzyme. It has been assumed that, when Tm is added to bacteria, tetrahydrofolates are depleted and the consequent lack of metabolites, which require derivatives of THF for their manufacture, prevent bacterial growth (27).

Tetrahydrofolates are used catalytically in bacteria for the manufacture of at least two amino acids (methionine and glycine), for purine synthesis, and for the initiation of protein synthesis (7). They are used in substrate quantities for the conversion of deoxyuridine monophosphate (dUMP) to deoxythymidine monophosphate (dTMP) by thymidylate synthetase (EC 2.1.1.b.). Miovic and Pizer (27) have suggested that, providing the thymidylate synthetase reaction is proceeding, Tm treatment will prevent the initiation of protein synthesis and those other biosyntheses which depend on catalytic quantities of THF derivatives. This view explains why thymineless (*thy*⁻) auxotrophs, which lack the enzyme thymidylate synthetase, can synthesize proteins at up to 80% of the normal rate during Tm treatment. A similar effect may be seen with prototrophs when their thymidylate synthetase is inhibited by 5-fluoro-2-deoxyuridine, in that protein synthesis can occur in the presence of Tm (27).

We have shown (J. Med. Microbiol., in press) that a prototrophic organism treated with Tm in a complex medium lacking any derivative of thymine behaved similarly to a thymineless mutant starved of thymine in a minimal medium, in that a rapid bactericidal effect was observed (2). In both cases, when thymine was added multiplication occurred. However, in this present work, when these experiments were repeated in minimal media the prototroph did not "die" rapidly in the presence of Tm nor was it able to multiply when thymine was added to the culture. We find, as did Angehrn and Then (1), that, provided certain metabolites (a purine, methionine, and glycine) are added, the prototrophic organism does die rapidly when treated with Tm in minimal medium. We additionally show, however, that these metabolites are not sufficient to allow complete recovery of the organism when thymine is added to the minimal medium. The additional requirement for thymine reversal of Tm-induced death in a minimal medium was investigated and found to be lysine. We propose a model based on the metabolism of THF to explain the differences in the requirements for thymineless death and Tm-induced death and their reversal by thymine derivatives in minimal media.

MATERIALS AND METHODS

Organisms. The bacteria used were *Escherichia coli* 114 (*thy*⁺) and its thymineless (*thy*⁻) mutant. This strain was used because of its high susceptibility to antibiotics and chemotherapeutic drugs. The other strain used was the methionineless auxotroph *E. coli*

K-12 (*met*⁻) 58.161/*sp* from W. Hayes (26). All three strains were maintained on slopes of oxoid blood agar base containing 60 μ g of thymine per ml and subcultured regularly.

Media. Davis-Mingioli (DM) minimal medium (15) was made up as described by Smith (32) and supplemented as indicated in the text; the carbon source was 0.28% glucose.

Reagents. 2-Deoxy-5-ribose, deoxyguanosine, xanthine, hypoxanthine, inosine, deoxyadenosine, and thymine were all purchased from Koch-Light, Colnbrook, Buckinghamshire, U.K., and sterile aqueous solutions were made by membrane filtration except for the last two, which were sterilized by autoclaving at 115 C for 20 min. Membrane filtration was used for sterilization of aqueous solutions of adenosine, adenine, guanine (B.D.H., Poole, Dorset, U.K.), vitamin-free Casamino Acids (Difco Laboratories, Detroit, Mich.), and the individual amino acids (Sigma Chemical Co., London, U.K.). Trimethoprim, as trimethoprim lactate, was kindly donated by the Wellcome Research Laboratories, and aqueous solutions were sterilized by membrane filtration. Results were expressed in terms of the concentration of the Tm base. Chloramphenicol was kindly donated by Parke-Davis, Detroit, Mich., and 5-fluoro-2-deoxyuridine was purchased from Calbiochem, San Diego, Calif. Both were sterilized by membrane filtration.

Tm treatment. Cultures were grown overnight at 37 C in DM medium containing 0.28% glucose plus other supplements where needed for the respective auxotrophic mutants. Exponential cultures were prepared by diluting a stationary-phase culture 1:20 in suitably supplemented, prewarmed DM medium at 37 C until the logarithmic phase was reached. The exponential culture was diluted, to give a final concentration of between 2×10^6 and 2×10^7 organisms per ml, in tubes containing DM medium plus the listed supplements and Tm. The tubes were incubated at 37 C, and the viable count was estimated by serial dilution in unsupplemented DM base and plated on oxoid MacConkey agar containing 60 μ g of thymine per ml. The plates were incubated at 37 C for 18 h and then counted.

RESULTS AND DISCUSSION

Conditions for a bactericidal response to Tm treatment in a minimal medium. In a complex medium lacking any thymine derivative, we have found that the prototroph *E. coli* 114 underwent thymineless death upon Tm treatment (*J. Med. Microbiol.*, in press). This effect was fully reversed when thymine or thymidine was added. However, when tested in DM medium, Tm was found to be only bacteriostatic to *E. coli* 114, and the addition of thymine had no significant effect on this response (Fig. 1). Thus, factors must be present in complex media which are responsible for (i) the bactericidal effect of Tm and (ii) its complete reversal by thymine.

There is a requirement for protein synthesis

FIG. 1. Effect of Tm on the viability of *Escherichia coli* 114 in DM. An exponential culture was diluted into prewarmed medium containing the listed supplements, and the viable count was followed. No additions, O; Tm (5 μ g/ml), ●; Tm (5 μ g/ml) and thymine (Thy) (60 μ g/ml), Δ.

during thymineless death (17, 28), and Tm reduces the level of cellular tetrahydrofolates which are required for the synthesis of some amino acids and for the initiation of protein synthesis. So DM containing all of the amino acids was tested to determine whether Tm could exert a bactericidal effect on *E. coli* 114, but no significant improvement was found (Fig. 2). However, when adenine as well as all of the amino acids were present in DM medium, Tm exerted a rapid bactericidal response, whereas adenine alone was without this effect. To investigate which amino acids are responsible for the bactericidal response, methionine, serine, and glycine, which are believed to require THF derivatives in their biosynthesis (7), were substituted for all of the amino acids and tested with adenine in DM medium. When Tm was added, this combination produced a rapid bactericidal response similar to that found when all of the amino acids and adenine were used (Fig. 2 and 3). Omission of methionine or glycine from the medium abolished this bactericidal effect, but when serine was omitted, the bactericidal response was still observed (Fig. 3). Further results showed that a lack of methionine or glycine, in the presence of all of the other amino acids, also failed to produce a bactericidal response. Hence, it can be concluded that the minimal requirements for Tm-induced death are methionine, glycine, and adenine.

FIG. 2. Effect of amino acids and adenine on the viability of Tm-treated *Escherichia coli* 114. An exponential culture was diluted into prewarmed medium containing Tm (5 $\mu\text{g/ml}$), adenine (Ad) (50 $\mu\text{g/ml}$), or all amino acids (AA) at 50 $\mu\text{g/ml}$ each. Symbols: $\bullet$, Tm; $\blacksquare$, Tm + Ad; $\blacktriangle$, Tm + AA; $\circ$, Tm + Ad + AA.

FIG. 3. Amino acid requirement for a bactericidal response of Tm treatment on *Escherichia coli* 114. An exponential culture was diluted into DM medium plus Tm and adenine plus the following amino acids at 50 $\mu\text{g/ml}$: methionine (Met) and serine (Ser) ($\blacksquare$); serine and glycine (Gly) ($\bullet$); methionine and glycine ($\square$); methionine, serine, and glycine ($\circ$).

This is similar to the requirements found by Angehrn and Then (1).

Effects of various purines and nucleosides on the action of Tm. Miovic and Pizer (27) found that, not only would adenine restore the

capacity for protein synthesis to Tm-inhibited cultures, but also that other purines or their nucleosides could be substituted for this base. Thus, it seemed possible that these compounds could replace adenine as a component essential in DM medium for Tm-induced death. This point was investigated experimentally, and the results (Table 1) show that there was little difference between the bactericidal capacities of adenine, deoxyadenosine, adenosine, guanine, deoxyguanosine, hypoxanthine, xanthine, or inosine when substituted for adenine in DM medium containing methionine and glycine. On the other hand, 2-deoxy-5-ribose did not produce a significant drop in viability. Since deoxyribonucleosides can provide bacteria with a deoxyribose-1-phosphate, which is known to affect bacterial thymine incorporation (8), all subsequent experiments were made with adenine, which is free from this defect.

Effect of chloramphenicol in Tm-mediated death. Classical thymineless death has a requirement for protein synthesis, without which bacteriostasis results (28). Angehrn and Then (1) have shown that protein synthesis occurs in minimal medium provided that glycine, methionine, and a purine nucleoside are added. Therefore, in order to test whether protein synthesis is similarly required for the bactericidal action of Tm, the effect of chloramphenicol (Cm) was investigated. It was found that, when Cm was added at the beginning of Tm treatment (Fig. 4), a bactericidal response occurred, albeit at a slower rate and with a great increase in the number of survivors. When Cm was added 30 min after the start of Tm treatment, the initial rate of death was similar to that found in the absence of Cm, but the duration of this phase was curtailed, giving an

TABLE 1. Viable count of a culture of *E. coli* 114 in DM medium^a

Further supplement at 50 $\mu\text{g/ml}$	Viable count at 7 h (organisms/ml)
None	1.82×10^6
Adenine	5.7×10^3
Deoxyadenosine	3.5×10^3
Adenosine	6.1×10^3
Guanine	6.8×10^3
Deoxyguanosine	1.2×10^4
Xanthine	7.7×10^3
Hypoxanthine	6.5×10^3
Inosine	8.9×10^3
2-Deoxyribose	1.1×10^6

^a Determined after 7 h of treatment with Tm (5 $\mu\text{g/ml}$) and with methionine and glycine (both at 50 $\mu\text{g/ml}$). The initial count was 6.3×10^6 organisms/ml.

FIG. 4. Effect of chloramphenicol (Cm) on the loss of viability of *Escherichia coli* during Tm treatment in the presence of methionine, glycine, and adenine. An exponential culture was diluted into prewarmed DM medium containing Tm, methionine, glycine, and adenine. Chloramphenicol (10 μ g/ml) was added as shown. Control, no chloramphenicol, ○; chloramphenicol added simultaneously with Tm, ■; chloramphenicol added 0.5 h after Tm, □.

intermediate survival level. Hence, protein synthesis appears to be required not only at the beginning but also during Tm treatment to produce a maximal bactericidal response.

Effects of Tm on thymineless mutants. Tm is not bactericidal to prototrophic organisms in plain DM medium, and this could result from a deficiency in protein synthesis arising indirectly from a lack of methionine, glycine, and adenine. To investigate this point further, the action of Tm on a thymineless mutant in minimal medium was studied. When Tm was added to the thymineless mutant in DM medium lacking thymine, it was found that a bactericidal effect occurred similar to that due to thymine starvation alone even though glycine, methionine, and adenine were absent (Fig. 5). Since protein synthesis is believed necessary for such a bactericidal effect (28), it would seem that Tm has no direct inhibitory effect on protein synthesis in this mutant. In support of this view, it was found that the rate of loss of viability was considerably reduced if Cm was added during thymine starvation irrespective of whether Tm was added. In other experiments, it was found that the bactericidal effect of thymine starvation was not increased by the addition of the amino acids and adenine; moreover, neither did

their addition increase the antibacterial effect of Tm on thymine starvation. Thus, the behavior of the thymineless mutant clearly differed from the prototroph in response to Tm in minimal medium. The former died rapidly without supplementation, whereas the latter died only when methionine, glycine, and adenine were added. It is possible that these differences may accrue from the absence and presence, respectively, of thymidylate synthetase. When this enzyme converts dUMP to dTMP, stoichiometric amounts of 5,10-methylene THF are oxidized to DHF, and hence the tetrahydrofolate levels are depleted. On the other hand, the thymineless auxotroph lacks this enzyme (3), and no major drain should occur to its levels of THF via this pathway. This being the case, the tetrahydrofolates in a thymineless mutant may be utilized for all catalytic roles, including the initiation of protein synthesis even when Tm is present.

To arrive at a similar situation with the prototrophic organism methionine, glycine and adenine must be supplied. These three metabolites may act in either of two ways. (i) Because their synthesis is catalytically dependent on tetrahydrofolates, their provision may release these tetrahydrofolates for the initiation of protein synthesis. It is known that purines regulate their own biosynthesis by feedback inhibition (18, 27), and hence their addition

FIG. 5. Effect of Tm on thymineless death of *Escherichia coli* 114 *thy*⁻. An exponential culture was diluted into DM medium lacking any thymine derivative and containing the following supplements: no additions (Δ); Tm (5 μ g/ml) (○); chloramphenicol (10 μ g/ml) (▲); Tm (5 μ g/ml) plus chloramphenicol (10 μ g/ml) (●).

could release 10-formyl THF and 5,10-methenyl THF into the THF pool. (ii) The provision of these metabolites may prevent the cellular production of 5,10-methylene THF, in which case oxidation of THF to DHF via thymidylate synthetase cannot occur. The former explanation seems insufficient because, if all biosynthetic pathways are competing for available THF, the addition of methionine, glycine, and adenine would simply release more THF for thymidylate biosynthesis. However, this would then deplete the THF levels and leave insufficient THF for the initiation of protein synthesis, which is necessary for Tm-induced death.

On the other hand, if the three metabolites prevented the formation of 5,10-methylene THF, consequently blocking the thymidylate synthetase reaction, a situation similar to thymine starvation of a thymineless mutant would arise. There would be an inability to produce thymine, and the resulting intact THF pool would provide the organism with the capacity for protein synthesis initiation. Thus, a response similar to thymineless death would occur in the presence of Tm.

Effect of 5-fluoro-2-deoxyuridine and one-carbon metabolites on Tm-mediated death. To investigate whether the drain of tetrahydrofolates through thymine synthesis is responsible for the differences between the *thy*⁺ and *thy*⁻ strains in response to Tm in DM medium, the inhibitor of thymidylate synthetase 5-fluoro-2-deoxyuridine (FUdR) (11) was used. FUdR

was added at various time intervals to the prototrophic bacterium during treatment with Tm in DM medium. To prevent degradation of this inhibitor to 5-fluorouracil, which can inhibit ribonucleic acid synthesis (22), deoxyadenosine was also added (36). It can be seen that a rapid bactericidal response was induced by FUdR (Fig. 6a) when added simultaneously with Tm. When FUdR was added 30 or 60 min after Tm, the rate of loss of viability was approximately the same although the number of ultimate survivors was considerably increased.

To compare whether FUdR could mimic the addition of methionine, glycine, and adenine, a similar kinetic experiment was made with the addition of these metabolites instead of FUdR. It can be seen (Fig. 6b) that similar bactericidal responses occurred to the prototroph when these metabolites were added during Tm treatment.

The agreement between these two experiments reinforces the view that the bactericidal response caused by Tm in minimal medium is dependent on the THF pool being conserved. When Tm is added to bacteria in unsupplemented DM medium, it would seem that the drug serves only to deplete the THF pool, thus leaving an insufficient level for protein synthesis which is essential for the bactericidal response (Fig. 4).

Tm treatment and methionineless auxotrophs. However, a previous report from this laboratory (29) may seem contradictory to

FIG. 6. Comparison of the effects of FUdR or methionine, glycine, and adenine addition to bacteria treated with Tm. An exponential culture was diluted into DM medium containing Tm at 5 µg/ml. In (a), FUdR (20 µg/ml) and deoxyadenosine (50 µg/ml) were added at the times shown. In (b), methionine, glycine, and adenine supplements (Sup.), each at 50 µg/ml, were added at the times shown. Control, Tm only, no additions, ○; addition simultaneously with Tm, ●; addition at 0.5 h after Tm, ■; addition 1 h after Tm, ▲.

these findings. When Tm treatment of a methionineless auxotroph, *E. coli* K-12 *met*⁻, was followed in minimal medium, it was found that methionine starvation increased the slow bactericidal effect of Tm. Similar experiments were repeated, and the results (Table 2) confirm these findings. However, when the *met*⁻ strain was incubated with Tm in the presence of adenine and all of the amino acids except methionine, the drop in viability was less than that observed when methionine was also present. Thus, this mutant, like *E. coli* 114, showed the sharpest drop in viability when methionine was present with glycine and adenine. However, providing adenine and glycine are absent, methionine starvation does increase the bactericidal effect of Tm in minimal medium. This may be due to a manifestation of methionineless death (10) when a methionineless auxotroph dies in the absence of methionine and, therefore presumably, of protein synthesis.

Nevertheless, the bactericidal response seen during methionine starvation of a *met*⁻ mutant in the absence of glycine and adenine does not compare with the kill observed when methionine, glycine, and adenine are present in minimal medium, which is likely to result from a response analogous to thymineless death.

Reversal of Tm action by thymine. The addition of thymine to a complex medium completely reversed the bactericidal effect of Tm on wild-type bacteria in that multiplication occurred. However, in DM medium the addition of thymine failed to promote multiplication of the same bacteria during Tm treatment (Fig. 1). Hence, there are metabolites in complex medium, other than thymine, that may reverse the effect of Tm on *thy*⁺ bacteria. Tm appears to have little effect on the growth of a *thy*⁻ auxotroph in the presence of thymine (Fig. 7)

TABLE 2. Effects of Tm treatment of *E. coli* K-12 *met*^{-a}

Time (h)	With adenine and all amino acids except methionine		No supplements	
	-Met	+Met	-Met	+Met
0	4.3×10^6	3.5×10^6	3.8×10^6	3.7×10^6
8	6.8×10^3	1.0×10^3	1.4×10^5	1.5×10^5
18	1.4×10^2	1.7×10^1	2.5×10^2	1.2×10^4

^a *E. coli* K-12 *met*⁻ was incubated with Tm in the presence or absence of methionine in DM medium. Samples supplemented with adenine and the remaining amino acids were also incubated. Samples were removed at 0, 8, and 18 h and serially diluted, and the viable count was determined.

FIG. 7. Reversal of Tm activity on *Escherichia coli* 114 *thy*⁻ by thymine addition. An exponential culture was diluted into DM medium containing the following supplements: thymine (60 μ g/ml) (O); thymine (60 μ g/ml) plus Tm (5 μ g/ml) (●); thymine (60 μ g/ml) plus chloramphenicol (10 μ g/ml) (■).

whether a minimal or complex medium is used (6, 33), just as it had little effect on thymine starvation (Fig. 5).

Previously, we suggested that Tm addition indirectly prevents protein synthesis by depleting the THF pool in *thy*⁺ bacteria. In the death experiments, this could be overcome by adding methionine, glycine, and adenine. Hence, vitamin-free Casamino Acids were tested with adenine to see whether their addition to DM medium could allow thymine to reverse the effects of Tm on a *thy*⁺ bacterium. The results (Fig. 8) showed that, when these metabolites were present in DM medium, the addition of thymine completely prevented the action of Tm in that multiplication occurred to an extent comparable to the control. These results were similar to those presented by Then and Angehrn (35) using thymidine. However, since these experiments were performed by using a mixture of all of the amino acids, further experiments were made to check whether methionine and glycine were the only amino acids responsible for this result. When methionine and glycine were used in place of the vitamin-free Casamino Acids, it was found (Fig. 9) that the rate of multiplication was significantly less than that of the control culture, and hence some other component, present in an amino acid mixture, is essential for maximal reversal of Tm action by thymine on *thy*⁺ bacteria.

To identify this compound, experiments were

FIG. 8. Reversal of Tm action on *Escherichia coli* 114 by thymine addition. Subcultures of an exponentially growing culture were diluted into prewarmed DM medium containing the following supplements: control, no additions (○); Tm (5 μ g/ml) plus thymine (60 μ g/ml) and vitamin-free Casamino Acids (CAA) (2 mg/ml) (■); Tm plus thymine (60 μ g/ml) plus adenine (50 μ g/ml) (▲); Tm (5 μ g/ml) plus thymine (60 μ g/ml) plus vitamin-free Casamino Acids (2 mg/ml) plus adenine (50 μ g/ml) (●).

made by using all of the amino acids tested in a crisscross pattern similar to that of Holliday (21). The results of these experiments revealed that lysine was the only amino acid which was additionally required in conjunction with methionine, glycine, adenine, and thymine to fully reverse the effects of Tm (Fig. 9).

Further experiments were performed that showed that adenine could be substituted by deoxyadenosine, guanine, deoxyguanosine, hypoxanthine, and inosine just as it could be in the death experiments. However, one of these purines or nucleosides had to be present to produce a multiplication of the *thy*⁺ organism in the presence of Tm.

DISCUSSION

Trimethoprim does not produce a bactericidal response when added to *E. coli* in a minimal medium, but it does produce a rapid bactericidal response in a complex medium lacking any thymine derivative (Amyes and Smith, J. Med. Microbiol., in press). We find that the addition of methionine and glycine plus a purine or purine nucleoside to DM medium is necessary

to produce a bactericidal response comparable to that obtained in a complex medium. Angehrn and Then (1) have shown that these supplements can restore the capacity for protein synthesis to bacteria being treated with Tm in minimal medium. We have amplified their findings by testing the effect of Cm during Tm treatment, which showed that sustained protein synthesis was essential for a maximal bactericidal response. Consequently, protein synthesis would seem to be indirectly inhibited by Tm when minimal medium, lacking methionine, glycine, and a purine, is used. By studying Tm treatment and thymine starvation of a *thy*⁻ mutant and the use of FUDR on the *thy*⁺ strain, we conclude that THF consumption by the action of thymidylate synthetase is responsible for this blockade in protein synthesis.

We suggest that, when bacteria are treated with Tm in a minimal medium, methionine, glycine, and adenine operate by preventing the formation of 5,10-methylene THF, the absence of which blocks the thymidylate synthetase reaction and the concomitant oxidation of THF. Figure 10 shows the interconversions of THF derivatives in the biosynthesis of one-carbon metabolites, and it can be seen that 5,10-methylene THF is the only substrate that can be oxidized to DHF. The addition of adenine will inhibit the purine biosynthetic pathway,

FIG. 9. Amino acid requirement for the reversal of Tm action in the presence of thymine and adenine. Subcultures from an exponential culture were diluted into DM medium containing Tm (5 μ g/ml), thymine (60 μ g/ml), and adenine (50 μ g/ml) plus the following amino acids at 50 μ g/ml each: all amino acids (AA) (●); methionine (Met) and glycine (Gly) (■); methionine, glycine, and lysine (Lys) (▲). The viable count is expressed on a logarithmic scale.

which utilizes 10-formyl THF and 5,10-methenyl THF (18, 27). Moreover, purines directly prevent the production of 5,10-methylene THF from 5,10-methenyl THF by feedback inhibition of 5,10-methylene THF dehydrogenase (13, 14, 31, 34). Thus, the addition of adenine will block the production of 5,10-methylene THF, and hence its oxidation to DHF via thymidylate synthetase will be reduced.

One of the two THF derivatives used in purine biosynthesis, 10-formyl THF, is also the precursor for *N*-formylmethionine, which is required for protein synthesis initiation (16). Thus, the addition of a purine should conserve 10-formyl THF for protein synthesis and prevent its depletion. However, purines alone were insufficient to produce enough protein synthesis for a bactericidal response during Tm treatment.

There are two other pathways (Fig. 10) by which THF, and hence 10-formyl THF, could be drained into the production of 5,10-methylene THF. These are the interconversions of serine to glycine and methionine to homocysteine. It was found that glycine, though not serine, was essential for a maximal bactericidal response. This is to be expected, since the supply of excess glycine to bacteria would prevent the direct formation of 5,10-methylene THF from THF, especially since the concentration of THF is reduced due to the inhibition of the dihydrofolate reductase reaction. Glycine is expected to push the reversible serine hydroxymethyl-

transferase reaction towards serine and THF, whereas the substitution of serine for glycine could reverse this reaction.

On the other hand, the role played by methionine in Tm-induced death is not as obvious as that of glycine (Fig. 10). Sibler and Mansouri (31) have shown that methionine represses serine hydroxymethyltransferase, an excess of which converts serine to glycine, and hence methionine should assist by preventing the formation of 5,10-methylene THF directly from THF. In addition, methionine controls its own biosynthesis by repressing 5,10-methylene THF reductase which would otherwise convert 5-methyl THF to 5,10-methylene THF (24, 30). Also, the equilibrium of the methyl transferase converting homocysteine to methionine is strongly in favor of methionine; therefore, the addition of methionine should not direct the flux of THF to 5-methyl THF and 5,10-methylene THF.

Thus, under the conditions of limited THF supply which are induced by Tm in a minimal medium, we postulate that methionine, glycine, and adenine will act to conserve the available THF levels by a concerted action preventing the formation of 5,10-methylene THF, as well as making up for their deficiencies produced by low THF levels. This deficiency would in turn affect, deleteriously, the thymidylate synthetase reaction; indeed, it has been shown that the incorporation of labeled carbon into thymine in media containing amino acids and purines is severely reduced by the addition of Tm (19). Thus, in minimal medium, prototrophic bacteria treated with Tm resemble a thymineless mutant being starved of thymine, provided the former, but not the latter, is supplied with methionine, glycine, and adenine. These supplements hence act to render the prototroph phenotypically into the thymineless state.

The foregoing explains why the thymineless death of a *thy*⁻ mutant can occur in a simpler medium than that required for a *thy*⁺ organism to be killed by the action of Tm.

A second difference between *thy*⁺ and *thy*⁻ bacteria is that, for thymine to completely reverse the bactericidal effect of Tm, the prototroph requires the addition not only of methionine, glycine, and adenine, but also lysine, to the medium. This requirement for lysine must be indirect, since its biosynthesis does not involve a THF derivative. It is known that lysine shares a common biosynthetic enzyme with methionine (25). Hence, it seems possible that high concentrations of added methionine could repress to some extent the synthesis of

FIG. 10. Interconversions of tetrahydrofolate intermediates and their relationship with purine and protein synthesis. Points of repression are indicated by T-bar and points of inhibition by T-bar . This reaction is virtually irreversible with the equilibrium strongly biased towards methionine and THF under normal conditions (★).

lysine. Although it would seem that the cellular availability of lysine is sufficient for the levels of protein synthesis necessary for Tm-induced death, insufficient amounts may be available for bacterial multiplication. It would seem likely that the extra demand for lysine during cell division stems from the requirement of its immediate precursor, diaminopimelic acid, in mucopeptide biosynthesis. Thus, excess methionine may reduce diaminopimelic acid availability to below that required for the normal rate of bacterial multiplication.

Thus, when methionine, glycine, adenine, and lysine are added to DM medium, thymine is able to restore growth to a *thy*⁺ organism being treated with Tm. However, thymine is not normally incorporated by *thy*⁺ bacteria (12, 23). Nevertheless, we have shown that, when Tm is present, the addition of methionine, glycine, and adenine can convert a *thy*⁺ bacteria into the phenotype of a *thy*⁻ mutant by preventing the thymidylate synthetase reaction. Thus, in the presence of these supplements, Tm treatment will allow sufficient dUMP to accumulate, so that its eventual catabolism to deoxyribose-1-phosphate will facilitate thymine uptake to a similar extent to that observed in *thy*⁻ mutants growing in otherwise unsupplemented minimal medium containing thymine (4, 5, 9). The remaining difference between *thy*⁻ and *thy*⁺ bacteria is the lysine requirement of the latter, which we postulate arises indirectly from the methionine supplementation and only occurs under conditions which promote bacterial multiplication.

ACKNOWLEDGMENTS

We are grateful to the Wellcome Trust for a Research Training Scholarship to S. G. B. A.

LITERATURE CITED

1. Angehrn, P., and R. Then. 1973. Nature of trimethoprim-induced death in *Escherichia coli*. *Arzneimittel-Forsch.* **23**:447-551.
2. Barner, H. D., and S. S. Cohen. 1954. The induction of thymine synthesis by T2 infection of a thymine requiring mutant of *Escherichia coli*. *J. Bacteriol.* **68**:80-88.
3. Barner, H. D., and S. S. Cohen. 1959. Virus-induced acquisition of metabolic function. IV. Thymidylate synthetase in thymine-requiring *Escherichia coli* infected by T₂ and T₄ bacteriophages. *J. Biol. Chem.* **234**:2987-2991.
4. Beacham, I. R., P. T. Barth, and R. H. Pritchard. 1968. Constitutivity of thymidine phosphorylase in deoxyriboaldolase negative strains: dependence on thymine requirement. *Biochim. Biophys. Acta* **166**:589-596.
5. Beacham, I. R., and R. H. Pritchard. 1971. The role of nucleoside phosphorylase in the degradation of deoxyribonucleosides by thymine-requiring mutants of *E. coli*. *Mol. Gen. Genet.* **110**:289-298.
6. Bertino, J. B., and K. A. Stacy. 1966. A suggested mechanism for the selective procedure for isolating thymine-requiring mutants of *Escherichia coli*. *Biochem. J.* **101**:32C-33C.
7. Blakley, R. L. 1969. The biochemistry of folic acid and related pteridines. North Holland, Amsterdam.
8. Boyce, R. P., and R. B. Setlow. 1962. A simple method of increasing the incorporation of thymidine into the deoxyribonucleic acid of *Escherichia coli*. *Biochim. Biophys. Acta* **61**:618-620.
9. Breitman, T. R., and R. M. Bradford. 1964. The induction of thymidine phosphorylase and excretion of deoxyribose during thymine starvation. *Biochem. Biophys. Res. Commun.* **17**:786-791.
10. Breitman, T. R., A. Finkleman, and M. Rabinowitz. 1971. Methionineless death in *Escherichia coli*. *J. Bacteriol.* **108**:1168-1173.
11. Cohen, S. S., J. G. Flaks, H. D. Barner, M. R. Loeb, and J. Lichtenstein. 1958. The mode of action of 5-fluorouracil and its derivatives. *Proc. Nat. Acad. Sci. U.S.A.* **44**:1004-1012.
12. Crawford, L. V. 1958. Thymine metabolism in strains of *Escherichia coli*. *Biochim. Biophys. Acta* **30**:428-429.
13. Dalal, F. R., and J. S. Gots. 1966. Inhibition of 5,10-methylene tetrahydrofolate dehydrogenase by purine nucleotides. *Biochem. Biophys. Res. Commun.* **22**:340-345.
14. Dalal, F. R., and J. S. Gots. 1967. Purification of 5,10-methylene-tetrahydrofolate dehydrogenase from *Salmonella typhimurium* and its inhibition by purine nucleotides. *J. Biol. Chem.* **242**:3636-3640.
15. Davis, B. D., and E. S. Mingioli. 1950. Mutants of *Escherichia coli* requiring methionine or vitamin B₁₂. *J. Bacteriol.* **60**:17-28.
16. Dickerman, H. W. 1971. The role of folate coenzymes in the initiation of protein synthesis. *Ann. N.Y. Acad. Sci.* **186**:70-81.
17. Ganguli, N., and S. B. Bhattacharjee. 1968. Influence of chloramphenicol on thymineless death in bacteria. *Biochim. Biophys. Acta* **169**:545-547.
18. Gots, J. S. 1957. Purine metabolism in bacteria. V. Feedback inhibition. *J. Biol. Chem.* **228**:57-66.
19. Harvey, R. J. 1973. Growth and initiation of protein synthesis in *Escherichia coli* in the presence of trimethoprim. *J. Bacteriol.* **114**:309-322.
20. Hitchings, G. H. 1969. Species differences among dihydrofolate reductases as a basis for chemotherapy. *Postgrad. Med. J.* **45**(Suppl.):7-10.
21. Holliday, R. 1956. A new method for the identification of biochemical mutants of micro-organisms. *Nature (London)* **178**:987.
22. Horowitz, J., and E. Chargaff. 1959. Massive incorporation of 5-fluorouracil into a bacterial ribonucleic acid. *Nature (London)* **184**:1213-1215.
23. Kammen, H. O. 1967. Thymine metabolism in *Escherichia coli*. I. Factors involved in utilization of exogenous thymine. *Biochim. Biophys. Acta* **134**:301-311.
24. Katzan, H. M., and J. M. Buchanan. 1965. Enzymatic synthesis of the methyl group of methionine. VIII. Repression-derepression, purification, and properties of 5,10-methylene tetrahydrofolate reductase from *Escherichia coli*. *J. Biol. Chem.* **240**:825-835.
25. Lederberg, J. 1950. Isolation and characterization of biochemical mutants of bacteria. *Methods Med. Res.* **3**:5-22.
26. Meynell, E., and N. Datta. 1966. The relation of resistance transfer factors to the F-factors (sex factor) of *Escherichia coli* K12. *Genet. Res.* **7**:134-140.
27. Miovic, M., and L. I. Pizer. 1971. Effect of trimethoprim on macromolecular synthesis in *Escherichia coli*. *J. Bacteriol.* **106**:856-862.
28. Pinney, R. J., and J. T. Smith. 1972. R factor elimination during thymine starvation: effects of inhibition of

- protein synthesis and readdition of thymine. *J. Bacteriol.* **111**:361-367.
29. Pinney, R. J., and J. T. Smith. 1973. Curing of an R factor from *Escherichia coli* by trimethoprim. *Antimicrob. Ag. Chemother.* **3**:670-676.
 30. Rowbury, R. J., and D. D. Woods. 1961. Further studies on the repression of methionine biosynthesis in *Escherichia coli*. *J. Gen. Microbiol.* **24**:129-144.
 31. Sibling, R., and A. Mansouri. 1971. Regulation of folate dependent enzymes. *Ann. N.Y. Acad. Sci.* **186**:55-69.
 32. Smith, J. T. 1967. Production of thymineless mutants in gram-negative bacteria (*Aerobacter*, *Proteus*). *J. Gen. Microbiol.* **47**:131-137.
 33. Stacy, K. A., and E. Simson. 1965. Improved method for the isolation of thymine-requiring mutants of *Escherichia coli*. *J. Bacteriol.* **90**:554-555.
 34. Taylor, R. T., H. Dickerman, and H. Weissbach. 1966. Control of one-carbon metabolism in a methionine-B₁₂ auxotroph of *Escherichia coli*. *Arch. Biochem.* **117**:405-412.
 35. Then, R., and P. Angehrn. 1973. Effects of trimethoprim on *Escherichia coli* under limited nutrition. *Arzneimittel-Forsch.* **25**:451-455.
 36. Yagil, E., and A. Rosner. 1971. Phosphorolysis of 5-fluoro-2'-deoxyuridine in *Escherichia coli* and its inhibition by nucleotides. *J. Bacteriol.* **108**:760-764.