Biochemical Pharmacology, Vol. 28, pp. 1467–1472.

0006-2952/79/0501-1467 $02.00/0

GROWTH OF THYMINE AUXOTROPHS ON SELECTED ANALOGS*

WILLIAM E. WHITE, JR. and MAUREEN D. DOLLINGER
Laboratory of Molecular Biology, University of Alabama in Birmingham, University Station, Birmingham, AL 35294, U.S.A

(*Received* 7 *August* 1978; *accepted* 7 *November* 1978)

Abstract—Thymine-requiring mutants of *Escherichia coli* and *Bacillus subtilis* will grow in the presence of sulfur-containing analogs of thymine and thymidine as the sole thymine-like source. Both 4-thiothymine and 4-thiothymidine promote exponential growth similar to that of their parent compounds, thymine and thymidine. Although the parent compound 5-bromouracil promotes growth, its sulfur-containing analog, 4-thio-5-bromouracil, does not. Additionally, the thymine-resembling compounds, 5-ethyluracil and 4-thio-5-ethyluracil, neither promote nor inhibit bacterial growth. 4(*O*-methyl) thymine oxime, a thymine analog with an oxime ether in place of oxygen at C-4, is both non-conducive and non-inhibitive to bacterial growth, and hence is not used by the cell as a thymine substitute. Although 4-thiothymine and 4-thiothymidine support bacterial growth, determination of the sulfur content of bacteria grown in the presence of these analogs shows that less than 1 per cent of the DNA still contains sulfur, thereby indicating that the sulfur atom is removed after entering the cell and probably prior to incorporation into DNA.

The effect of thymine deficiency on thymine-requiring strains of *Escherichia coli* was first described by Cohen and Barner [1], who found DNA synthesis to be blocked. They found that thymine auxotrophs of *E. coli* grown on thymine-deficient medium had lost their colony-forming ability. When certain analogs of thymine were added to the auxotrophs, growth occurs.

Various thymine analogs have been shown to incorporate in place of thymine into the DNA of bacteria and other cells. Hybrid DNA was produced when 5-bromouracil was substituted for thymine in the absence of protein and RNA syntheses [2]. Other substituted uracil compounds which resemble thymine have been used in measuring the growth of human lymphocytes and fibroblasts in culture [3] and of mouse melanoma cells [4]. In the mouse cells it was found that 5-ethyldeoxyuridine and 5-bromodeoxyuridine enter cellular DNA and incorporate to the extent of 12 and 18 per cent of total thymine respectively [4]. 5-Iododeoxyuridine also has been reported to be incorporated in DNA, while 5-fluorodeoxyuridine has been reported as not incorporated [5]. It is presumed that the larger halogen atoms, bromine and iodine, being similar in size to the methyl group of thymine, would incorporate, while the fluorine atom, being smaller, would not incorporate [5].

Other work with purine analogs has shown 5-thioguanine to be incorporated into the DNA of tumorous tissue [6]. Work also has been performed in leukemic cells to test incorporation of the sulfur-containing analogs 6-mercaptopurine and 6-thioguanine into the cells, and to test subsequent sensitizing of the cells to a second compound having a selective affinity for these analogs [7].

Work done with 4-thiouridine (S^4UR) added exogenously demonstrates that this uridine analog inhibits the growth of various microbial and mammalian cells in culture. It is presumed that because of the cytotoxic activity of S^4UR, this analog competes with UR and TdR for various enzymes in the biosynthesis of RNA and DNA [8]. It has been suggested that the cytotoxic effects of thiopyrimidine analogs are related to interference with RNA synthesis [9]. 4-Thiothymidine, another thiopyrimidine, was first suggested as a four-substituted pyrimidine nucleotide analog by Lezius and Scheit [10]. They suggested that the substitution of sulfur in place of oxygen at position C-4 of thymidine, although probably weakening the hydrogen bonding, would have a comparably small change in the structure of the molecule. In their work, 4-thiothymidine-triphosphate was substituted for thymidine-triphosphate as substrate for DNA polymerase.

In our studies, 4-thiothymine incorporation into bacterial DNA in the absence of thymine was measured. By observing overall growth, it can be determined whether the thymine analog is being utilized during DNA synthesis, and by monitoring the sulfur content the amount of analog incorporation can be measured. The oxime ether, 4(*O*-methyl) thymine oxime, was also tested as a thymine analog. Due to the size and isoelectric similarities between the oxygen atom of the thymine and the nitrogen atom of the oxime, similar H—O and H—N bonds were anticipated. This is demonstrated in Fig. 1. The top portion of Fig. 1, a and b, shows the two possible conformations of 4(*O*-methyl) thymine oxime. The bottom portion of the figure shows the hypothesized maintenance of the Watson–Crick base pairing between one form of the oxime ether with the purine adenine (c) and the other oxime ether form with the purine guanine (d). Because of the restricted rotation of the sp^2N about the double bond in 4(*O*-methyl) thymine oxime, two isomers can be discerned. The syn-isomer would allow for greater similarity to thymine and hence possible Watson–Crick pairing, since the unshared electrons of this isomer are on the same side as N-3. The anti-isomer, however, would have a lesser tendency toward incorporation because of the steric hindrance caused by the position of its methoxy group.

*This work was supported in part by American Cancer Society Institutional grant IN-66M.

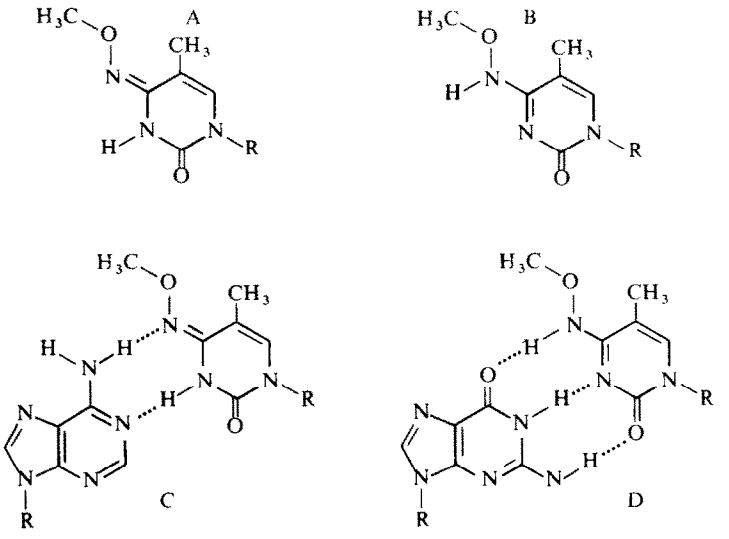

Fig. 1. A and B describe the two conformations of 4(*O*-methyl) thymine oxime. C and D describe the hypothesized bonding of one form of the thymine oxime with adenine (C) and the other form of the thymine oxime with guanine (D).

MATERIALS AND METHODS

Bacterial strains and media

The *E. coli* χ-216 (thy^-) and *E. coli* χ-15 wild type strains were obtained from the Department of Microbiology of the University of Alabama in Birmingham Medical Center. The *Bacillus subtilis* 168 (thy^-, trp^-) strain was obtained from R. G. Wake, Department of Biochemistry, University of Sydney, Australia.

Initial tests for purity of the *E. coli* (χ-216) strain utilized nonenriched minimum medium containing: 12 g Tris, 2 g KCl, 2 g NH_4Cl, 0.5 g $MgCl_2 \cdot H_2O$, 0.02 g Na_2SO_4, 0.05 g NaH_2PO_4, 7.5 ml concentrated HCl, 5 g glucose and 1 liter H_2O. The minimum medium used for *B. subtilis* was as above, with the addition of 20 mg tryptophan. Enriched minimum medium, also used to test purity, contained the above minimum medium solutions plus 40 μg/ml of thymine. Each strain was plated on both enriched and nonenriched minimum medium, allowed to incubate overnight, and was observed the next morning. Strains grew well on enriched minimum medium and not at all (even over a 3-day period) on nonenriched minimum medium.

Synthesis of analogs

Preparation of 4-thiothymine. 4-Thiothymine was prepared by the method of Ueda and Fox [11]. A solution containing 5.0 g thymine (0.044 mole) and 4.2 g P_2S_5 (0.019 mole) in 110 ml pyridine was refluxed for 3 hr. The solution became yellow immediately, and turned orange after 20 min. The upper layer was separated from the dense oily residue and evaporated to dryness with a rotary evaporator to give a bright yellow solid. Paper chromatography and infrared spectroscopy indicated the absence of thymine. The final product was recrystallized from water. Ultraviolet λ_{max} (DMF)* = 337 nm. Lit. = 338 [12].

* DMF, dimethylformamide.

Preparation of 4-thiothymidine. 4-Thiothymidine was prepared from thymidine by the method of Scheit [13] by a series of reactions involving acetylation of the 3′ and 5′ hydroxyl groups with acetic anhydride, refluxing with P_2S_5 in pyridine, and hydrolysis of the ester bonds in dioxane/ammonia at room temperature overnight. The final product was purified on a silica gel column using chloroform/MeOH.

Preparation of 5-ethyluracil. 5-Ethyluracil was prepared in a series of reactions by the methods of Gauri *et al.* [14]. (a) Preparation of 5-ethylbarbituric acid: to 110 ml ethanol were added 2.3 g sodium (0.1 mole), 18.8 g diethyl ethylmalonate (0.1 mole) and 6 g urea (0.1 mole). After refluxing for 17 hr, the solution was filtered while warm and washed with EtOH, giving 14.8 g (83%) of the 5-ethylbarbituric acid monosodium salt. (b) Preparation of 5-ethyl-6-chlorouracil: 10 g of the 5-ethylbarbituric acid sodium salt (0.056 mole) was refluxed for 1.5 hr in 50 ml $POCl_3$ containing 1 ml H_3PO_4. All of the solid had dissolved after 45 min. The excess $POCl_3$ was distilled under reduced pressure and the remaining solution was poured into ice. The solid was filtered and recrystallized from $EtOH/H_2O$, giving 2.0 g of product. Another 3.7 g of product could be recovered following evaporation of the mother liquor, giving a total yield of 9.7 g (97%). M.p. (water) = 213°. Lit. 213–215° [15]. (c) Preparation of 5-ethyluracil: 1 g of 5-ethyl-6-chlorouracil (5.7 m-moles) was suspended in 60 ml of 0.02 M NaOH and 20 ml of MeOH containing a catalytic amount of 10% Pd/C. H_2 pressure was maintained at 40 lb/in^2 for 6 hr. The resulting suspension was filtered and the solid extracted with dimethylformamide. Evaporation afforded 0.3 g of 5-ethyluracil (40%). M.p. < 280°. Lit. 298–300° (decomp) [15]; i.r. (KBr disk) 3300–3000 (N—H, C—H); 2850 (C—H); 1750, 1680 (C = O) cm^{-1} plus other.

Preparation of 4-thio-5-ethyluracil. A solution of 5-ethyluracil (0.070 g, 0.5 m-mole) and P_2S_5 (0.1 g) was

refluxed overnight in 5 ml pyridine in a 10-ml round bottom flask. The liquid was decanted and the oil extracted with another 5 ml of hot pyridine. The two pyridine fractions were combined and evaporated to dryness in a beaker in the hood. Recrystallization from 10 ml MeOH and 15 ml EtOH gave 0.055 g (70%) of 4-thio-5-ethyluracil. Additional product could be recovered by evaporation of the alcohol. M.P. < 275°. Ultraviolet λ_{max} (DMF) = 336 nm ($\varepsilon = 1.3 \times 10^4$) i.r. (KBr) 3450 (N—H); 3200 (?); 3100 (C—H); 2900–2950 (C—H); 1720, 1640 cm^{-1} plus other.

Preparation of 4-thio-5-bromouracil. Although this synthesis was not completely satisfactory, we obtained better yield and a cleaner product by converting uracil to 4-thiouracil in pyridine and then brominating the 4-thiouracil in DMF, rather than converting the bromouracil to its thio derivative as done with the other compounds. A yield of about 60 per cent could be obtained from the oily residue produced by the preferred method. The product could be recrystallized from water. Ultraviolet (DMF) $\lambda_{max} = 333$ nm ($\varepsilon = 2.6 \times 10^4$).

Preparation of 5-methyl-N^4-methoxycytosine. 4-Thiothymine was dissolved in 1 M methoxyamine and the pH adjusted to 7.0 with NaOH. The reaction required about a week for the 335 band to disappear. Ultraviolet λ_{max} (water) = 273 nm ($\varepsilon = 2.3 \times 10^4$) and 237 nm ($\varepsilon = 2.2 \times 10^4$). Anal. Calc. for $C_6H_9N_3O_2$: C, 46.45; H, 5.85; N, 27.08. Found: C, 46.12; H, 5.88; N, 26.59.

Preparation of 5-methyl-N^4-methoxycytidine. 5-Methyl-N^4-methoxycytidine was prepared by the method of Iida *et al.* [16]. Two g of methoxyamine hydrochloride (24 m-moles) and 0.23 g of 4-thiothymidine (0.9 m-mole) were dissolved in 50 ml of water, and the pH was adjusted to 6.8 with NaOH. After 3 days, the absorbance at 335 nm had disappeared. The water was then evaporated and the product purified on silica gel thin-layer chromatography, using 7% MeOH in chloroform. Extraction from the silica gel gave 0.17 g of 5-methyl-N^4-methoxycytidine, an oil (71%).

Analysis of analogs

Compounds used in biological studies were diluted with water to 7.3×10^{-3}M concentrations (5-methyl-N^4-methoxycytosine and 5-methyl-N^4-methoxycytidine to 1.0×10^{-4} M concentrations) and then chromatographed on a Perkin–Elmer 601 syringe pump HPLC, equipped with a variable wavelength detector, using a 15% octadecalsulfate reverse phase column. Samples were first run on a 0–100% acetonitrile/water gradient and then run at a certain % acetonitrile indicated by the graident. All compounds were easily separated from their precursors. Compounds eluted as single peaks except for thiothymidine. The thiothymidine contained a small impurity which has not been identified, but which is neither thymidine nor thymine, as its absorbance at 335 indicates it to be a 4-thio derivative.

Growth determination

Bacteria were grown from a single colony overnight in a Difco bacto-tryptone broth—the nutrient medium used for all bacteria. The next morning the bacteria were harvested, washed twice with saline and suspended in a 0.9% saline solution. Culture tubes were then prepared with 5 ml of nonenriched minimum medium, a given aliquot of the bacteria–saline suspension, and 0.1 ml of one of the 7.3×10^{-3} M thymine analog solutions. As controls, a culture tube with nonenriched minimum medium and bacteria, with no analog added, and a culture tube with bacto-tryptone broth and bacteria were prepared. When growth was exceedingly slow, bacteria were grown overnight in each respective analog-enriched minimum medium to either logarithmic phase or maximum stationary phase. The next morning 1 ml of this overnight suspension was added to fresh analog-enriched minimum medium and allowed to resume growth. This procedure allows for a decrease in otherwise long lag phases, and establishes all cultures in logarithmic phase [17]. All of the culture tubes were then placed in a water bath equipped with a shaker at 37°, and aliquots were plated onto bacto-tryptone agar plates at approximately 2-hr intervals. It should be noted that for those times that the bacteria were allowed to grow in the water bath for over 10 hr, plating was also done on nonenriched minimum medium to check for possible contamination or mutation to a strain no longer requiring thymine. Bacteria were scored the next day, measuring cell survival by their colony-forming ability. As an alternate method, growth was followed by optical density readings (660 nm, 1 cm path length) in a Gilford 2400-2 spectrophotometer. Here, growth rate was determined by a graph of optical density time.

Incorporation of thio derivatives

Bacteria were grown in 1 liter of minimum medium enriched with 20 mg of 4-thiothymidine or 4-thiothymine as the only thymine-like source for 4 days in a 37° water bath with aeration.

Cells were harvested by centrifugation at 5000 rev/min for 20 min at room temperature, washed in 50 ml of saline–EDTA buffer (0.15 M NaCl; 0.1 M EDTA; pH 8), centrifuged again for 10 min at 5000 rev/min and suspended in 30–35 ml of saline–EDTA. Isolation of bacterial DNA was by the procedure of Marmur [18], with the cell suspension lysed with 2 ml of 25% sodium lauryl sulfate, followed by a 10-min incubation at 60°. After cooling to room temperature, the solution was treated with 2 ml of SSC buffer (0.015 M

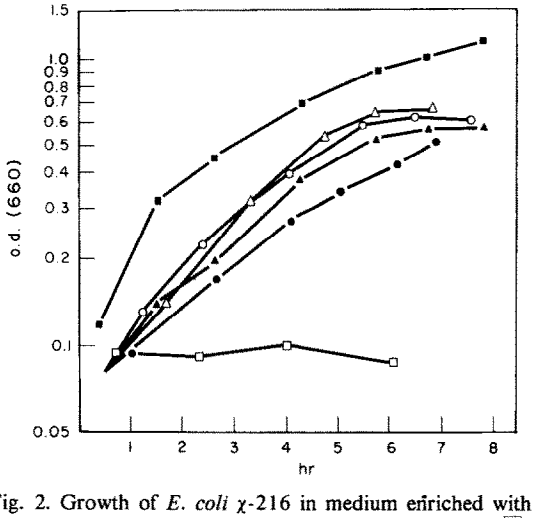

Fig. 2. Growth of *E. coli* χ-216 in medium enriched with various thymine analogs plotted against time. Symbols: (□) none, (○) thymine, (●) 4-thiothymine, (△) thymidine, (▲) 4-thiothymidine, and (■) bactotryptone.

sodium citrate; 0.15 M NaCl), for 30 min, followed by 10 ml of 5 M sodium perchlorate and, finally, approximately 50 ml of a chloroform–isoamyl alcohol (24:1) solution, as described by Marmur. The solution was stirred for 20 min and then separated into three layers by centrifugation at 5000 rev/min for 10 min. The aqueous layer was removed from the protein layer and the chloroform layer, and this protein extraction was repeated. The final aqueous layer was treated with RNAse T_1 (50 μl) and pancreatic RNase (0.1 ml) for 30 min in a 37° water bath. Deproteinization was performed one more time to remove final traces of protein. The final solution was dialyzed for 24 hr in phosphate buffered saline at 5° (0.025 mole $Na_2HPO_4 \cdot 7H_2O$; 0.025 mole $NaH_2PO_4 \cdot H_2O$; 0.15 mole NaCl; pH 7.2) to remove the RNA, and sulfur content was measured by absorbance readings at 335 nm. DNA concentration was determined at 260 nm.

RESULTS

Growth of bacteria in the prescence of thymine, thymidine, 4-thiothymine and 4-thiothymidine

As demonstrated in Fig. 2, thymine auxotrophic bacteria will grow exponentially in the presence of either thymine (T), thymidine (TdR), 4-thiothymine (S^4T) or 4-thiothymidine (S^4TdR) as the only thymine-like source. As inferred from this figure, T, TdR and S^4TdR analogs produce relatively similar growth rates, while S^4T, although similar to the others, contributes to somewhat slower growth. For comparison, bacteria grown in bacto-tryptone broth are also included, and the bacto-tryptone growth curve appears similar to those of the four thymine analogs. For further comparison, Fig. 3 shows that nonenriched minimum medium will begin to reduce bacterial viability over time. This process is known as thymineless death. By comparing the decrease in the viability of cells grown on nonenriched medium to the increase in viable cells grown on thymine-enriched medium (Fig. 3), a more detailed description can be ascertained for the general growth of the four thymine analogs (Fig. 2).

Growth of bacteria in the prescence of oxime ether

There is no apparent growth of either *B. subtilis* (168) or *E. coli* (χ-216) when 1.0×10^{-4} M 4-methyl-N^4-methoxycytosine or 5-methyl-N^4-methoxycytidine is substituted for thymidine in minimal growth medium. Additionally, as shown in Fig. 4, 1.0×10^{-4} M 5-methyl-N^4-methoxy-cystosine does not inhibit growth of *B. subtilis* when this oxime is added with 40 μg/ml of thymidine to the minimal growth medium. In this case, the base form of the oxime was used with the nucleoside thymidine in order to minimize any possibility of competitive inhibition that might be caused by the simultaneous use of two nucleosides. If the two nucleosides, 5-methyl-N^4-methoxycytidine and thymidine, were in fact used, competition for the same site on the DNA molecule might occur, and, ultimately, this might have an effect on the overall results.

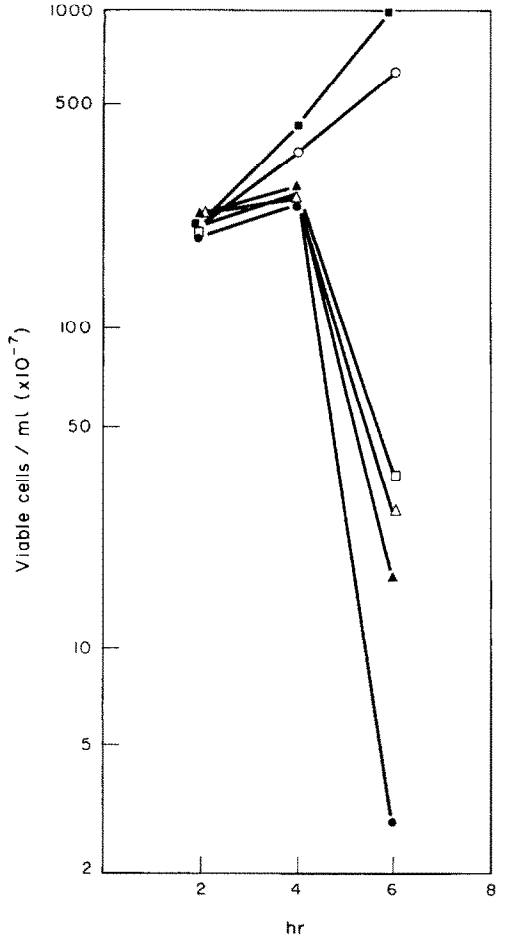

Fig. 3. Growth of *E. coli* χ-216 in medium enriched with various thymine analogs plotted against time. Symbols: (○) 5-bromouracil, (●) 4-thio-5-bromouracil, (△) 5-ethyluracil, (▲) 4-thio-5-ethyluracil, (□) no analog—nonenriched medium, and (■) thymine.

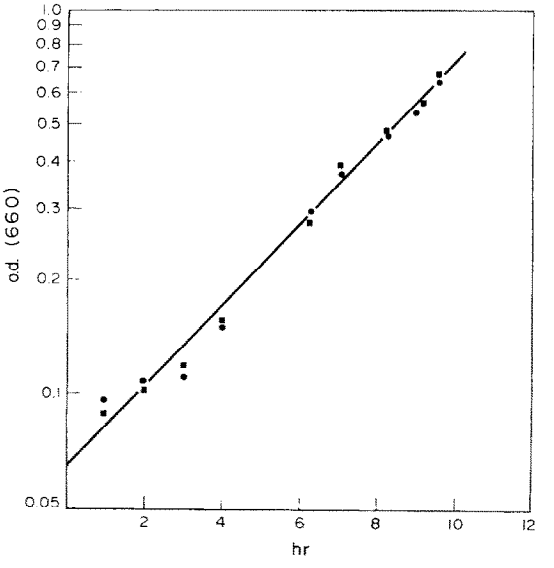

Fig. 4. Growth of *B. substilis* 168 bacteria in medium enriched with thymidine or enriched with thymidine and 4(*O*-methyl) thymine oxime. Symbols: (●) thymidine, and (■) thymidine + 5-methyl-N^4-methoxycytosine.

Growth of bacteria in the presence of 5-ethyluracil, 4-thio-5-ethyluracil, 5-bromouracil and 4-thio-5-bromouracil

As mentioned previoulsy, 5-bromouracil has been shown to incorporate into DNA in place of thymine. Duplication of these results are shown in Fig. 3, as demonstrated by an increase in bacterial colony number. However, the sulfur-containing analog, 4-thio-5-bromouracil, appears to promote a decrease in the number of viable colonies, a result similar to thymineless death. Two other thymine-resembling analogs, 5-ethyluracil and 4-thio-5-ethyluracil, produce a similar decrease in viable colony number. Hence, any one of these three different thymine analogs, when used as the sole thymine source available to the thymine-requiring cells, does not promote bacterial growth but instead demonstrates bacterial growth resembling that of unenriched medium.

Figure 5 demonstrates the results of an additional test designed to determine whether or not the thymine analogs described in Fig. 3 actually cause the decrease in colony number through inhibition of bacterial growth. As can be seen in Fig. 5, the wild-type bacteria (*E. coli* χ-*15*), which do not require an outside source of thymine, grow equally well whether or not the thymine analogs 5-ethyluracil or 4-thio-5-ethyluracil are present. The overall bacterial behavior with respect to media enriched with either 4-thio-5-bromouracil 5-ethyluracil or 4-thio-5-ethyluracil, from Figs. 3 and 4, appear to resemble that of enriched minimal medium—as if no analogs are present.

Determination of sulfur content of bacterial DNA

B. subtilis was grown in a liter of minimum medium enriched with 20 mg of tryptophan and 20 mg of 4-thiothymidine. The DNA was obtained by the method described previously. DNA concentration was determined spectroscopically at 260 nm and 4-thiothymidine concentration at 335 nm, using ε_{335} = 15,700 [12]. Less than 1 per cent of the nucleotides contained the 4-thio substituent. Similar results were obtained from DNA isolated from *E. coli* grown on 4-thiothymine. Sodium perchlorate (6 M) had no effect on the u.v. spectrum of S^4T over a 2-hr period.

DISCUSSION

Both 4-thiothymine and 4-thiothymidine promote growth of thymine auxotrophic bacterial cells. As seen in Fig. 2, bacteria enriched with thymine, thymidine or 4-thiothymidine grow the fastest, followed by bacteria enriched with 4-thiothymine. These results indicate the relative ease with which a sulfur-containing nucleoside, 4-thiothymidine, is utilized in the cell. The slower growth with 4-thiothymine may result from differences in membrane permeability for enzyme specificities. As seen in Fig. 3, thymineless death occurs in the nonenriched minimum medium control. By use of the plating method, bacterial viability was shown to decrease markedly; this is due to the absence of thymine or any thymine analog.

When two substitutions are made on uracil, so that 4-thio-5-bromouracil and 4-thio-5-ethyluracil are formed, incorporation does not appear to occur. As seen in Fig. 3, the parent compound, 5-bromouracil, is utilized by the thymine auxotrophic bacteria; this tends to indicate incorporation into the DNA. However, the parent compound, 5-ethyluracil, will not incorporate into DNA, nor will the 4-thio-5-bromouracil and 4-thio-5-ethyluracil compounds. In further tests to determine if 5-ethyluracil or 4-thio-5-ethyluracil inhibits wild-type bacterial growth, it has been demonstrated that these two analogs show no effect on growth. As shown in Fig. 5, bacterial growth in medium enriched with 5-ethyluracil or 4-thio-5-ethyluracil is the same as growth in nonenriched minimum medium.

The DNA isolated from thymine auxotrophic bacteria grown in 4-thiothymine-enriched medium contained

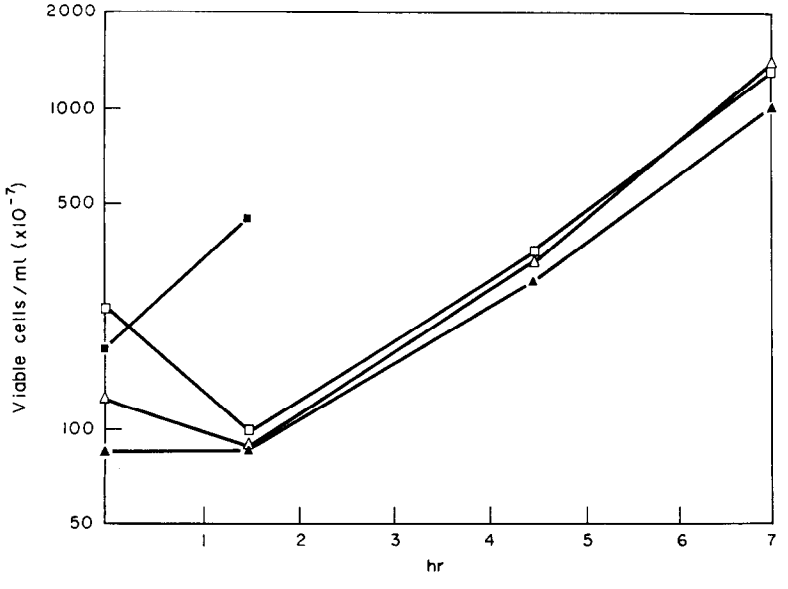

Fig. 5. Growth of wild-type bacteria in medium enriched with various thymine analogs plotted against time. Symbols: (△) 5-ethyluracil, (▲) 4-thio-5-ethyluracil, (□) no analog—unenriched medium, and (■) bactotryptone.

less than 1% 4-thiothymine. Hence, somewhere along the metabolic pathway the sulfur at C-4 is removed and replaced, probably with oxygen, thereby producing the usual substrate—thymine. 4-Thiothymine appears to be reasonably stable in aqueous solution because no hydrolysis was observed during autoclaving of an aqueous solution at 120° for 30 min, nor by perchlorate during workup. Hence, thymine probably does not result from uncatalyzed hydrolysis of 4-thiothymine.

The oxime ether, 4(*O*-methyl) thymine oxime, is neither conducive nor inhibitory to bacterial growth and probably is not utilized as a thymine replacement because of the large methoxy group. This absence of growth on minimal medium enriched with the oxime ether analogs indicates that the bacteria cannot convert these compounds to the normal substrates.

The oxidation of certain thiopyrimidines to their oxoanalogs is known to be catalyzed by certain enzymes [19]. The enzymatic removal of sulfur moieties from drugs has some pharmacological consequences. First, if the sulfur atom plays the principal role in drug action, perhaps the drug may be destroyed before incorporation by removal and/or replacement of the sulfur atom (as appears to be the case for the thio-analogs of thymine), thereby rendering the drug ineffective. On the other hand, if the sulfur atom is not essential, or if the sulfur atom would be preferentially removed after entrance into the cell, then perhaps the drug action may be enhanced. The sulfur atom could perhaps aid in membrane penetration or solubility of the drug and, once within the cell, could be replaced by a more therapeutically effective group, thereby forming the desired drug compound that could not easily enter the cell on its own. Recognition of this instability of the sulfur atom could lead to preferential development of sulfur-containing drugs that would be more effective with the sulfur atom lost or replaced within the cell. Hence, the determination of the role of the sulfur atom for a particular drug could be very useful in predicting the mode of action of the drug and subsequent effectiveness.

REFERENCES

1. S. S. Cohen and H. D Barner, *Proc natn. Acad. Sci. U.S.A.* **40**, 885 (1954).
2. P. C Hanawalt, O. Maaloe, D. J. Cummings and M. Schaechter, *J. molec. Biol.* **3**, 156 (1961).
3. S. Singh, I. Willers, H. W. Goedde and K. K. Gauri, *Hum. Genet.* **24**, 135 (1974).
4. S. Silagi, R. F. Balint and K. K. Gauri, *Cancer Res.* **37**, 3367 (1977).
5. C. Heidelberger, *Prog. nucleic Acid Res. molec. Biol.* **4**, 1 (1965).
6. G. A. LePage, *Cancer Res.* **20**, 403 (1960).
7. R. A. Roosa and E. D. DeLamater, *Cancer Res.* **20**, 1543 (1960).
8. T. I. Kalman, A. Bloch, G. L. Szekeres and T. J. Bardos, *Biochem. biophys. Res. Commun.* **55**, 210 (1973).
9. C. B. Lozzio and P. W. Wigler, *J. cell Physiol.* **78**, 25 (1971).
10. A. G. Lezius and K. H. Scheit, *Eur. J. Biochem.* **3**, 85 (1967).
11. T. Ueda and J. J. Fox, *J. med. Chem.* **6**, 697 (1963).
12. R. W. Lamon, *J. heterocyclic Chem.* **5**, 837 (1968).
13. K. H. Scheit, *Chem. Ber.* **101**, 1141 (1968).
14. K. K. Gauri, K. W. Pflughaupt and R. Muller, *Z. Natur.* **245**, 833 (1969).
15. T. D. Kulikowski and D. Shugar, *Acta biochim. pol.* **18**, 209 (1971).
16. S. Iida, K. C. Chung and H. Hayatsu, *Biochim. biophys. Acta* **308**, 198 (1973).
17. M. Frobisher, *Fundamentals of Microbiology*, p. 211. W. B. Saunders, Philadelphia (1957).
18. J. Marmur, *J. molec. biol.* **3**, 208 (1961).
19. L. L. Poulsen, R. M. Hysolp and D. M. Ziegler, *Biochem. Pharmac.* **23**, 3431 (1974).