Biochemical Pharmacology, Vol. 24, pp. 1003–1006. Pergamon Press. 1975. Printed in Great Britain.

# THE BIOCHEMICAL BASIS OF THE ANTIMICROBIAL ACTION OF SULFONAMIDES AND TRIMETHOPRIM *IN VIVO*—II.

## EXPERIMENTS ON THE LIMITED THYMINE AND THYMIDINE AVAILABILITY IN BLOOD AND URINE

RUDOLF THEN and PETER ANGEHRN

Department of Experimental Medicine, F. Hoffmann-La Roche & Co. Ltd., Basle, Switzerland

(*Received* 2 *July* 1974; *accepted* 17 *October* 1974)

**Abstract**—*Escherichia coli* 15T$^-$, which undergoes "thymineless death" upon thymine deprivation, is not viable in human blood and urine. The addition of as little as 0·05 μg/ml thymidine results in a distinct growth promoting effect in both fluids whereas the thymine concentrations needed for growth are higher. Growth kinetics with thymine are different from those observed with thymidine. By comparison of the growth of *E. coli* 15T$^-$ in synthetic media with that in blood and urine it is assumed that the concentrations of thymidine and thymine in the biological fluids are below 0·05 μg/ml and 0·2 μg/ml, respectively. These concentrations are not sufficient to antagonize the bactericidal action of trimethoprim, sulfonamides or their combinations. From the growth characteristics of *E. coli* 15 (TAU)$^-$ in blood it is concluded that other pyrimidines such as uracil (or uridine) are present and that there is a specific lack of thymine and thymidine. The *in vivo* conditions for the action of antifolates are discussed.

Antifolates such as trimethoprim and the sulfonamides lead to a drastic reduction in the viable cell count of bacteria in synthetic media which are free of thymine (T) and thymidine (TdR) [1, 2]. It was previously shown [3] that these compounds act bactericidally in human blood and urine and it was assumed that the absence of T and TdR is responsible for this effect and perhaps for any antibacterial effect which occurs at therapeutic concentrations.

In this respect T and TdR seem to behave quite differently from other sulfonamide antagonists, which are obviously present in biological fluids in sufficient concentrations to antagonize the particular deficiencies [4–6]. It is known that exogenously administered TdR is rapidly incorporated into DNA and metabolized, resulting in an extremely short half-life in mice and rats [7–11]. There are, however, few data available on the concentrations of T and TdR in biological fluids, and especially in man [7, 12, 13].

In this paper we have attempted to determine T and TdR concentrations in blood and urine with the aid of the thymine auxotroph *Escherichia coli* 15T$^-$. Whereas thymine- auxotrophs, normally obtained by routine methods [14, 15] require high concentrations of thymine, this strain responds to low concentrations depending on a second mutation in the deoxyribose catabolism [16]. Growth kinetics experiments show that using this strain no T or TdR was detected, and concentrations in blood and urine are below 0·05 and 0·2 μg/ml, respectively.

### MATERIAL AND METHODS

*Strains and culture conditions.* We are grateful to Professor Mennigmann (Frankfurt) for providing *Escherichia coli* 15T$^-$ and *E. coli* 15 (TAU)$^-$. *E. coli* B was used for the experiments with trimethoprim. The strains were maintained on agar slants (Difco blood agar base) and transferred to minimal medium, as described in the preceding paper [3]. For growth of *E. coli* 15T$^-$ 2 μg/ml of thymine was added, and for growth of *E. coli* 15 (TAU)$^-$ 10 μg/ml each of arginine, uracil and thymine, or, if growth occurred in casamino acid containing medium, only thymine and uracil were added. The mutant strains were washed thoroughly by millipore filtration before inoculation of the particular medium.

Citrated human blood from stored samples was heated at 56° for 30 min before use to destroy complement, which otherwise would lead to a rapid destruction of the bacterial strains used. The TdR content of blood is not influenced by heat treatment [12]. Urine from several healthy persons was collected in the morning, pooled and the pH adjusted to 7·0 with solid NaOH. After centrifugation it was sterilized by filtration.

*Chemicals.* Uracil, thymine and thymidine were obtained from Merck (Darmstadt). These substances were tested by NMR-spectroscopy and microanalysis and proved to be of high purity (>99%). 2-Deoxyadenosine and inosine were purchased from Fluka (Buchs SG) and arginine from Calbiochem (Los Angeles).

### RESULTS

*Some growth characteristics of* E. coli *15T$^-$ in synthetic media. E. coli* 15T$^-$ responds to withdrawal of T with cell death, known as "thymineless death" [see e.g. ref 17]. The death rate depends on the generation time and therefore on the composition of the growth

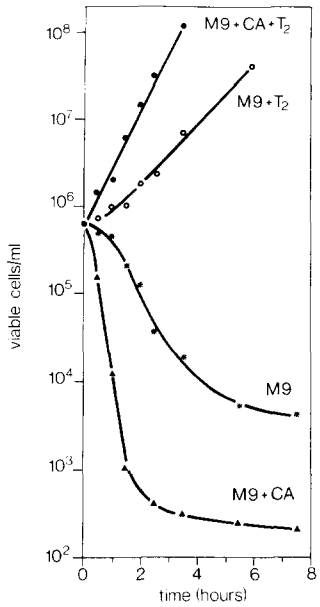


Fig. 1. Thymineless death of *E. coli* 15T⁻ in minimal salts medium M9 and in M9, supplemented by 0·1% casamino acids (M9 + CA), and its prevention by T (2 μg/ml).

medium. In amino acid containing medium the inactivation rate is higher than in minimal salts medium (Fig. 1).

Thymineless death can be prevented by T or TdR. Limiting concentrations, which do not allow growth, result in slower inactivation and higher survival rates [18]. Growth kinetics in the presence of T or TdR differ strikingly (Fig. 2). Whereas low TdR concentrations led to transient control-like growth without lag, limiting T-concentrations did not prevent cell death but led to a considerably higher survival rate depending on the T-concentration.

No attempt has yet been made to find out whether the short growth promoting action of TdR is stopped by complete exhaustion or by the cleavage of TdR by thymidine phosphorylase [19].

The concentrations of T and TdR required to prevent cell death vary according to the growth medium. Whereas, in medium M9, about 0·04 μg/ml T prevents death entirely, 0·3 to 0·4 μg/ml are necessary after supplementation with casamino acids. Such figures cannot be determined exactly for TdR, owing to the completely different growth kinetics on TdR. The TdR-concentrations needed are also higher in the casamino acid containing medium.

*Growth behaviour of* E. coli *15T⁻ in blood and urine.* When exponentially growing cells of *E. coli* 15T⁻ were thoroughly washed and transferred to urine, treated as described under Methods, cell death occurred after a certain lag. This lag was due partially to the medium change, but could not be completely abolished by preculturing in urine, supplemented with T or TdR. Cell

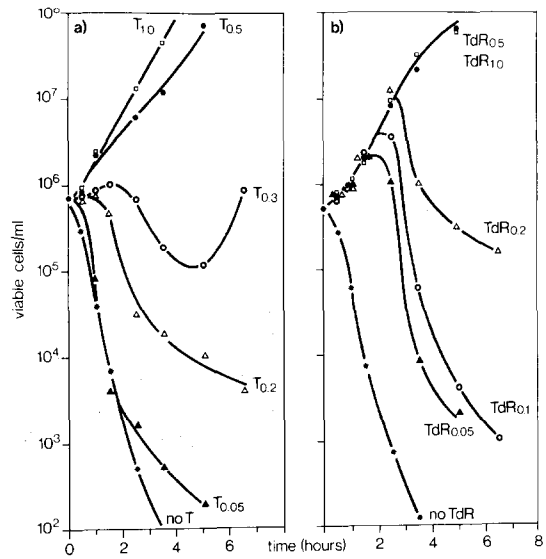


Fig. 2. Thymineless death of *E. coli* 15T⁻ in minimal salts medium M9, supplemented by 0·1% casamino acids, and the effects of limiting concentrations of (a) T (0·05, 0·2, 0·3, 0·5 and 1·0 μg/ml), and (b) TdR (0·05, 0·1, 0·2, 0·5 and 1·0 μg/ml).

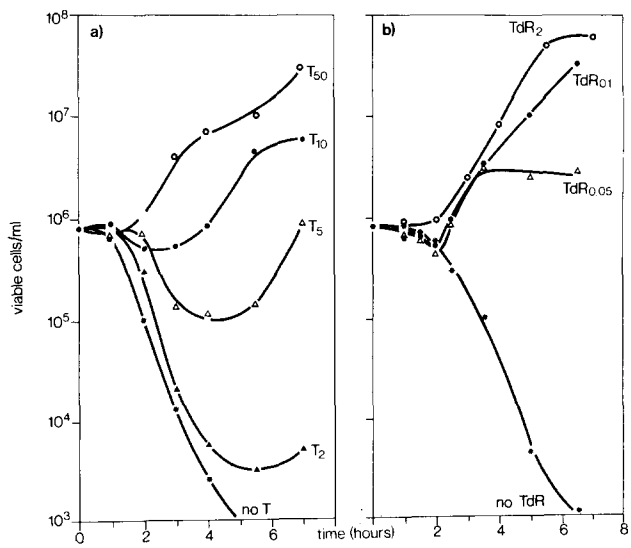


Fig. 3. Thymineless death of *E. coli* 15T⁻ in urine. Effect of limiting concentrations of added (a) T (2, 5, 10, 50 μg/ml) and (b) TdR (0·05, 0·1 and 2 μg/ml).

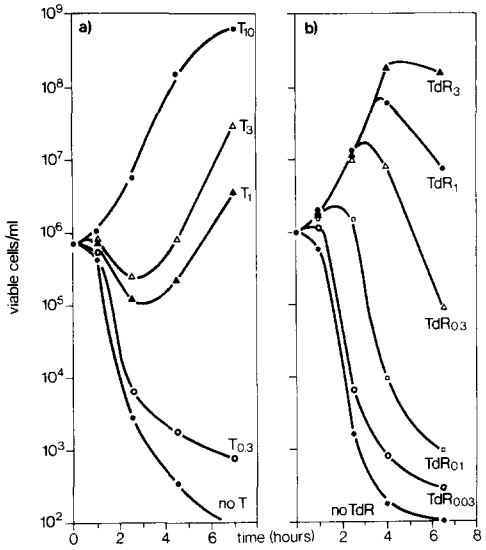


Fig. 4. Thymineless death of *E. coli* 15T⁻ in human blood. Effect of limiting concentrations of added (a) T (0·3, 1, 3, and 10 μg/ml) and (b) TdR (0·03, 0·1, 0·3, 1, and 3 μg/ml).

death was prevented by the addition of low TdR-concentrations, but only by high T-concentrations (Fig. 3). Similar results were obtained when transfer to human blood occurred (Fig. 4).

The great effectiveness of TdR in preventing cell death as seen in synthetic medium, may justify the assumption that concentrations are below 0·05 μg/ml in blood and urine. The application of the same assumption to the T-concentration seems to be more difficult. It is known that the T-availability in *E. coli* 15T⁻ is strongly influenced by a variety of compounds present in blood and other biological fluids [20, 21]. However, in *E. coli* 15T⁻ such inhibiting agents can be antagonized by the addition of deoxyadenosine (AdR)

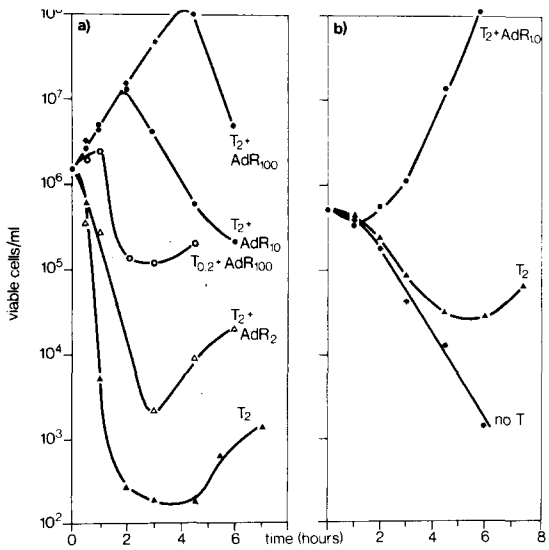


Fig. 5. (a) Bactericidal effect of trimethoprim (2 μg/ml) on *E. coli* B in M9 medium supplemented by 0·1% casamino acids and 30 μg/ml inosine. Enhanced availability of added T (0·2 and 2 μg/ml) by increasing concentrations of AdR (2, 10, 100 μg/ml). (b) Thymineless death of *E. coli* 15T⁻ in urine. Restoration of the T-availability (2·0 μg/ml T) by AdR (1·0 μg/ml).

[22]. High AdR-concentrations enable a TdR-like action of T in antagonizing trimethoprim-inhibition in *E. coli* B as seen in Fig. 5a. The low effectiveness of T in supporting growth of *E. coli* 15T⁻ observed in urine was greatly enhanced by the addition of AdR (Fig. 5b).

Concentrations below 0·2 μg/ml of T were detectable in this way. The experiment demonstrates that T utilization by *E. coli* 15T⁻ is inhibited in these fluids. Addition of AdR to blood and urine alone, however, revealed no growth supporting properties. T-concentrations therefore seem to be below 0·2 μg/ml.

The preceding paper has shown that TdR is more effective than T in antagonizing trimethoprim-induced T-deficiency. From the experiments with *E. coli* 15T⁻ it seems that the T or TdR concentrations required to overcome a "bactericidal action" are not present in these fluids. The possibility as to whether the utilization of T during trimethoprim-induced T-deficiency could be impaired by uridine was investigated. In the presence of 200 μg/ml uridine, the concentration of T needed for 50 per cent growth on 0·5 μg/ml TM was six times higher than that needed in its absence.

*Selective lack of T and TdR.* In a few experiments the growth kinetics of *E. coli* 15 (TAU)⁻ were checked in blood. Besides T this strain, derived from *E. coli* 15T⁻, requires arginine and uracil for growth, and cell death occurs if only arginine and uracil are present [17]. The growth and inactivation behaviour of this strain in synthetic medium and blood (Fig. 6) may lead one to assume the presence of a pyrimidine that meets the uracil requirement of this strain. That would mean the existence of a selective lack of T and TdR but not of other pyrimidines in blood. No attempt has yet been made to find an explanation for the initial lag in the growth of the controls growing with uracil and T.

## DISCUSSION

There is little information on the T and TdR content in biological fluids, especially in man [7, 10, 12, 13, 23].

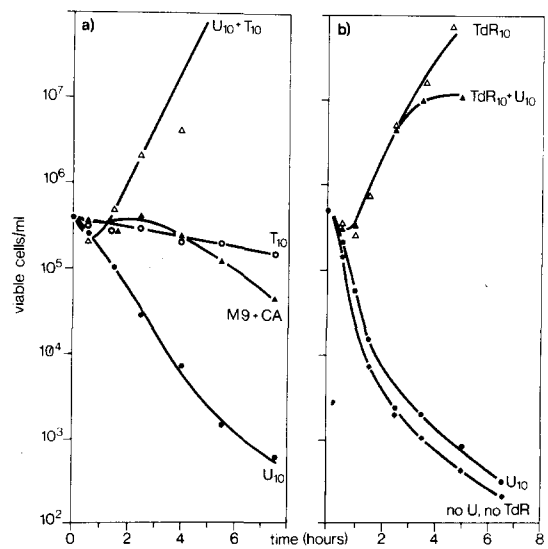


Fig. 6. (a) Growth response of *E. coli* 15 (TAU⁻) to addition of T, uracil or both (10 μg/ml each) in M9 + CA medium (see Fig. 1). (b) Growth response of *E. coli* 15 (TAU)⁻ in human blood to addition of uracil, TdR or both (10 μg/ml each).

Both substances, however, play a key role for the action of all antifolates *in vivo* against either bacteria or protozoa [24]. Some conclusions may be drawn from the growth kinetics experiments.

*E. coli* 15T$^-$ was unable to grow in blood and urine. A comparison of the effects of TdR added to these fluids and to synthetic media, with consideration of different generation times, leads to the assumption that TdR concentrations seem well below 0·05 μg/ml. This concentration is far below the level reported for mice and rats [10, 12]. The varying effectiveness of different concentrations of T and TdR in blood, urine and synthetic media is mostly due to different generation times in the particular fluids, since the T- and TdR-requirement is a function of the growth rate. On account of the characteristic growth promoting effect of TdR on *E. coli* 15T$^-$, already described, minute quantities would have been detected, if any had been present.

Conclusions regarding the T-content of those fluids depend on more sophisticated considerations. It is known that rather low concentrations of certain pyrimidine and purine nucleosides inhibit T-utilization in the strain used [20, 21]. Such effects have been demonstrated in urine. The addition of AdR, however, reveals low T-concentrations, which were not detected in blood and urine. It seems reasonable to conclude that the concentration of T in these fluids is below 0·2 μg/ml and therefore unable to antagonize the action of TM or sulfonamides. The inhibition of T-utilization during TM-induced T-deficiency, as demonstrated for uridine, does not seem to play any role, because of the high concentrations needed. In any case, such an effect would support the bactericidal action of antifolates. Those inhibitory effects are not to be expected with TdR [21].

The application of the results to *in vivo* conditions seems reasonable, although it is not yet certain whether the T or TdR levels in the blood change during storage. Such changes, however, should be specific for T and TdR, since other pyrimidines, e.g. uracil or uridine, were detected with *E. coli* 15 TAU$^-$. This precaution seems to be less important for urine, as fresh specimens were used, but the question of changes in the T and TdR concentrations will be further investigated.

Recently, Ferone *et al.* [25] demonstrated the presence of a thymidine phosphorylase in horse erythrocytes and its absence in the blood of other animals and man. Changes in the TdR-content of human blood by this enzyme may therefore be excluded. Another aspect seems to be of considerable interest. Co-trimoxazole- (a combination of trimethoprim and sulfamethoxazole) resistant strains have recently been isolated from several patients. These strains, mostly isolated from urine, were thymine-auxotrophs [26, 27]. The question whether the T- or TdR-concentration in the urine of such patients is elevated, remains open.

Thy$^-$-mutants occur rather frequently under aminopterin or trimethoprim treatment, a widespread selective technique for isolating such mutants [14]. If infectious processes increase the amount of T or TdR in body fluids and tissues, one would expect a high frequency of such mutants and even therapy failures. In line with our assumption, the occurrence of such T-auxotrophs, however, seems to be a very rare event and has only recently been demonstrated, though the causative agent Co-trimoxazole has been used extensively for several years. Our results refer to *E. coli* and may be generalized to cover a variety of strains in which thymineless death has been demonstrated; these are mostly but not exclusively gram negative strains. Some pathogens, however, may turn out to be more resistant to T-deprivation.

*Acknowledgments*—We acknowledge with gratitude the skilful experimental work of Mrs. L. Deschang and Mrs. U. Payne.

## REFERENCES

1. P. Angehrn and R. Then, *Arzneimittel-Forsch.* **23,** 447 (1973).
2. P. Angehrn and R. Then, *Chemotherapy* **19,** 1 (1973).
3. R. Then and P. Angehrn, *Biochem. Pharmac.* **23,** 2977 (1974).
4. M. Allgöwer, *Helv. physiol. pharmacol. Acta* **2,** 569 (1944).
5. B. Weissmann, P. A. Bromberg and A. B. Gutmann, *J. biol. Chem.* **224,** 407 (1957).
6. P. L. Altman and D. S. Dittmer, *Blood and Other Body Fluids.* Fed. Am. Soc. Exp. Biol. Bethesda (1971).
7. N. Gross and M. Rabinowitz, *Biochim. biophys. Acta* **157,** 648 (1968).
8. J. Fulcrand, J. Bisconte and R. Marty, *C. R. Soc. Biol.* **162,** 1584 (1968).
9. G. Lonngi and M. Gonzalez-Diddi, *Arch. Invest. med.* **3,** 123 (1972).
10. W. L. Hughes, M. Christine and B. D. Stollar, *Analyt. Biochem.* **55,** 468 (1973).
11. J. R. Rubini, E. P. Cronkite, V. P. Bond and T. M. Fliedner, *J. clin. Invest.* **39,** 909 (1960).
12. W. C. Schneider, *J. biol. Chem.* **216,** 287 (1955).
13. J. J. Jaffe, J. J. McCormack and E. Meymarian, *Biochem. Pharmac.* **21,** 719 (1972).
14. J. B. Bertino and K. A. Stacey, Biochem. J. **101,** 32c (1966).
15. H. Nakamura, *J. gen. Microbiol.* **68,** 235 (1971).
16. T. R. Breitman and R. M. Bradford, *Biochim. biophys. Acta* **138,** 217 (1967).
17. S. S. Cohen, *Ann. N.Y. Acad. Sci.* **186,** 292 (1971).
18. C. E. Deutch and C. Pauling, *J. Bact.* **106,** 197 (1971).
19. M. Rachmeler, J. Gerhart and J. Rosner, *Biochim. biophys. Acta* **49,** 222 (1961).
20. R. M. Behki and S. N. Lesley, *Can. J. Microbiol.* **19,** 485 (1973).
21. D. Freifelder, *J. Bact.* **90,** 1153 (1965).
22. J. R. Beacham, K. Beacham, A. Zaritsky and R. H. Pritchard, *J. molec. Biol.* **60,** 75 (1971).
23. J. E. Cleaver in *Thymidine metabolism and Cell Kinetics. Frontiers of Biology* (Ed. A. Neuberger and E. L. Tatum) Vol. 6, p. 60. North Holland, Amsterdam (1967).
24. V. E. Reid and M. Friedkin, *Molec. Pharmac.* **9,** 74 (1973).
25. R. Ferone, S. R. M. Bushby, J. J. Burchall, W. B. Moore and D. Smith, *Abstr. 8th Int. Cong. Chemother.* Vol. A, No. A-203. (1973).
26. J. Barker, D. Healing and J. G. P. Hutchinson, *J. clin. Path.* **25,** 1086 (1972).
27. O. A. Okubadejo and R. M. Maskell, *J. gen. Microbiol.* **77,** 533 (1973).