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

ACTION OF SULFONAMIDES AND TRIMETHOPRIM IN BLOOD AND URINE

RUDOLF THEN and PETER ANGEHRN

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

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Abstract—Trimethoprim (2 μg/ml), sulfamethoxazole (5 μg/ml) and a combination of these compounds reduced the viable cell count of *Escherichia coli* in human blood and urine in a bactericidal manner. This bactericidal action can be prevented by thymidine or thymine, the effectiveness of thymidine being considerably greater than that of thymine. Growth kinetics in the presence of trimethoprim, thymidine or thymine differed strikingly. Concentrations of thymidine below 0·05 μg/ml were detectable by their growth promoting effect, which started without a lag. Considerably higher concentrations of thymine, however, did not initially prevent the onset of cell inactivation, but resulted in a higher level of survivors. It is concluded that the concentrations of thymidine and thymine in blood and urine are too low to prevent a bactericidal action of trimethoprim, sulfonamides or a combination of these whereas other compounds, such as amino acids and purines, which support the bactericidal action, are present in sufficient quantities.

BOTH sulfonamides and trimethoprim (TM) interfere with tetrahydrofolic acid biosynthesis in bacteria and some protozoa. The application of substances that interfere in the biosynthesis of a small molecule is an unique case in chemotherapy. The great majority of all known antimetabolites cannot be used for the treatment of infections because of the presence of antagonizing agents in biological fluids. Demonstration of the effectiveness of sulfonamides *in vivo* clearly leads to the assumption that insufficient concentrations of *p*-aminobenzoic acid are present in the body[1] to antagonize their action. Likewise, most pathogens are unable to use extracellular folic acid compounds and cannot therefore overcome the imposed block by the uptake of folic acid present in sufficient quantities in the host fluids.[2]

It is possible, however, to bypass the inhibited steps in a noncompetitive manner by the uptake of those metabolites, the synthesis of which depends on tetrahydrofolate cofactors. *Streptococcus faecalis*, which is not able to synthesize folic acid,[3] grows well in the absence of folic acid if purines, amino acids and thymine are available.[4–6] Similarly it has been shown that the action of sulfonamides and TM is overcome by certain antagonists.[7,8] Most of these experiments were performed in synthetic media or in commercially available complex media. In the present investigation, the bactericidal effects of sulfonamides and TM or a combination of these compounds in biological fluids, viz. blood and urine, were studied and compared with the effects observed in synthetic media.[9–11]

MATERIALS AND METHODS

Strains and culture conditions. We are grateful to Professor Mennigman, Frankfurt, for providing *Escherichia coli* B and *E. coli* 15, the parenteral strain of *E. coli* 15T$^-$. The strains were kept on agar slants (Difco blood agar base) and transferred weekly to minimal salts medium M9.[12] For the experiments exponentially growing cells subcultured daily were used.

Urine was collected from several healthy persons in the morning and pooled. The pH was adjusted to 7·0 with solid NaOH, the urine centrifuged and sterilized by filtration.

Human citrated blood, from stored samples, was heated to 56° for 30 min before being used to destroy complement.

To follow growth kinetics, cells were grown in 30 ml of undiluted urine or blood in 100 ml Erlenmeyer flasks in a shaking water bath at 37°. Additions were made as indicated. Inocula were adjusted to about 10^6 cells/ml, or, in the experiments with sulfonamides, to 10^4–10^5 cells/ml.

At intervals indicated, samples of 1 ml were taken, diluted appropriately and plated on Difco blood agar base. Colonies were counted after 24 hr at 37°.

Chemicals. Sulfamethoxazole (used as sodium salt) and trimethoprim were Roche products. Thymine, thymidine, adenosine-5′-monophosphate, adenosine-5′-diphosphate and adenosine-5′-triphosphate were purchased from Merck (Darmstadt), inosine from Fluka (Buchs, SG) and adenosine from Calbiochem (Los Angeles).

RESULTS AND DISCUSSION

Bactericidal effect of sulfonamides in blood and urine. Growth kinetics of *E. coli* B in blood and urine revealed bactericidal properties of sulfamethoxazole (SMZ) which could be antagonized fully by thymidine (TdR) (Fig. 1). This is in accordance with

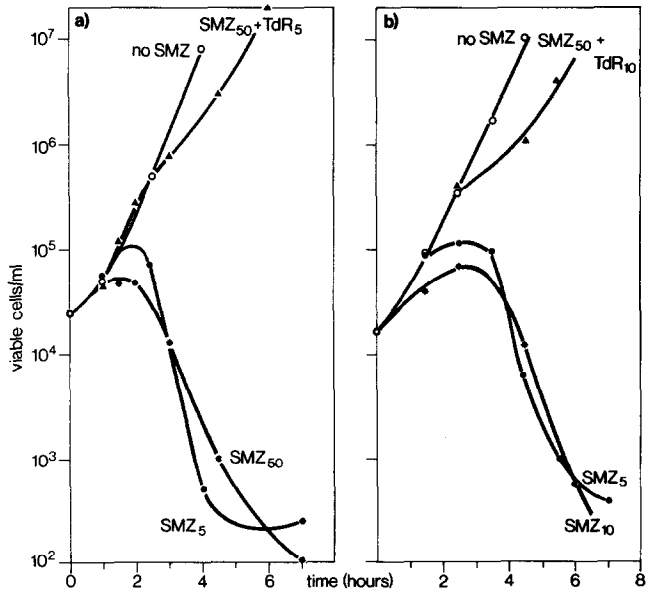

FIG. 1. Bactericidal effect of SMZ (5,10,50 μg/ml) on *E. coli* B in human blood (a) and in urine (b) and its antagonization by TdR (5,10 μg/ml).

the previously described action of sulfonamides in appropriately supplemented synthetic media.[13] In contrast to the action of TM, there is a certain lag between the addition of sulfonamide and the onset of cell death, due to the mode of action of sulfonamides.[14]

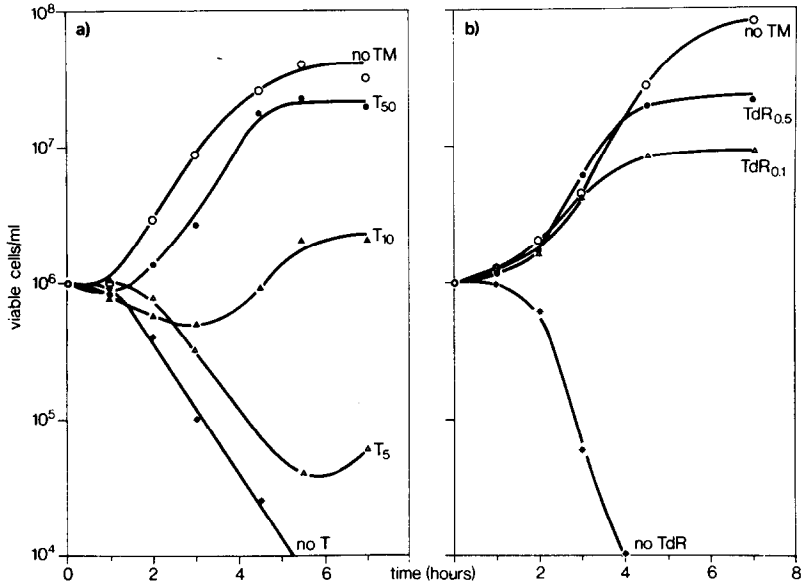

FIG. 2. Bactericidal effect of TM (2 μg/ml) on *E. coli* 15 in urine and its antagonization by (a) various concentrations of T (5,10,50 μg/ml) or (b) by TdR (0·1, 0·5 μg/ml). TM, T and TdR added at time zero.

As recently pointed out, there does not seem to be any strict relationship between sulfonamide concentration and bactericidal efficacy.[11] Low sulfonamide concentrations, easily obtained with therapeutic doses, are effective.

Bactericidal effect of TM, alone and combined with sulfamethoxazole in human blood and urine. In urine, low concentrations of TM caused an abrupt drop in the viable cell count of *E. coli* 15 (Fig. 2). Similar behaviour was seen in human blood. As shown in Fig. 3, this applies also to a combination of SMZ and TM, which is extensively used in the chemotherapy of infections.[15]

The slower inactivation rate in urine is due to the slower growth in this medium, which also results in a longer generation time. The considerable lag observed in urine before onset of growth or cell death is due partially to the medium change (cells were precultured in minimal medium M9), but could not be completely abolished by pregrowing the cells in urine.

As has been shown in synthetic media,[9–11] the bactericidal effect can be completely antagonized by addition of thymine (T) or TdR. Growth kinetics with limiting concentrations of T and TdR differ greatly in these fluids as is seen in Figs. 2 and 3. Concentrations of 0·1 μg/ml TdR or less will prevent cell death for some time in all media. T-concentrations required for the same effect are nearly 100 times as high.

The same differences can be shown in a synthetic medium containing amino acids and a purine source (Fig. 4). The presence of minute quantities of TdR (below 0·05 μg/ml) results in transient uninhibited growth. Cell death begins after a certain

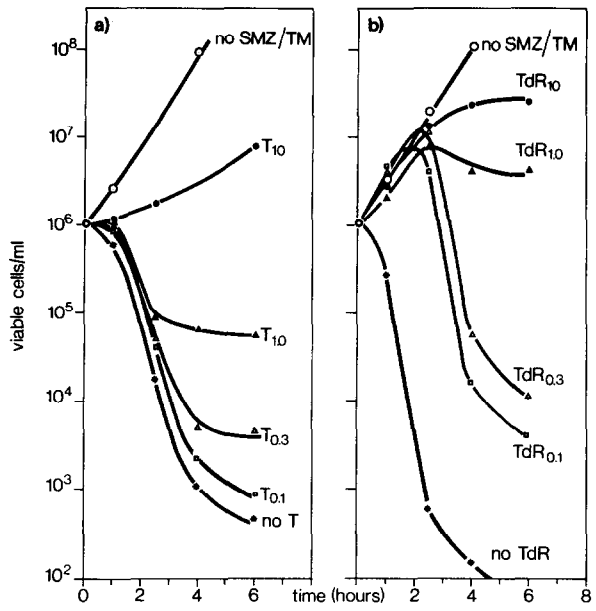

FIG. 3. Bactericidal effect of a combination of SMZ and TM (5 μg/ml SMZ and 1 μg/ml TM) added at time zero on *E. coli* 15 in human blood. (a) Effect of different concentrations of T (0·1, 0·3, 1·0, 10 μg/ml). (b) Effect of different concentrations of TdR (0·1, 0·3, 1·0, 10 μg/ml).

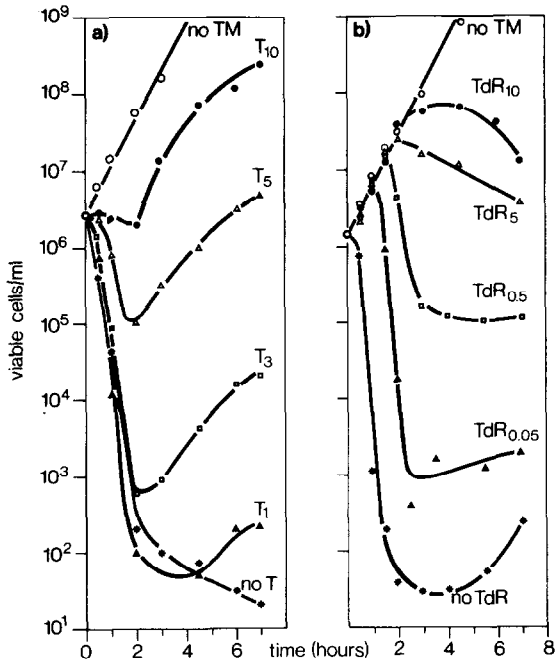

FIG. 4. Bactericidal effect of TM (2 μg/ml) on *E. coli* B in minimal salts medium M9, supplemented by 0·1% Casamino acids and 30 μg/ml inosine. (a) Antagonistic effect of different concentrations of T (1, 3, 5, 10 μg/ml). (b) Antagonistic effect of different concentrations of TdR (0·05, 0·5, 5·0, 10 μg/ml).

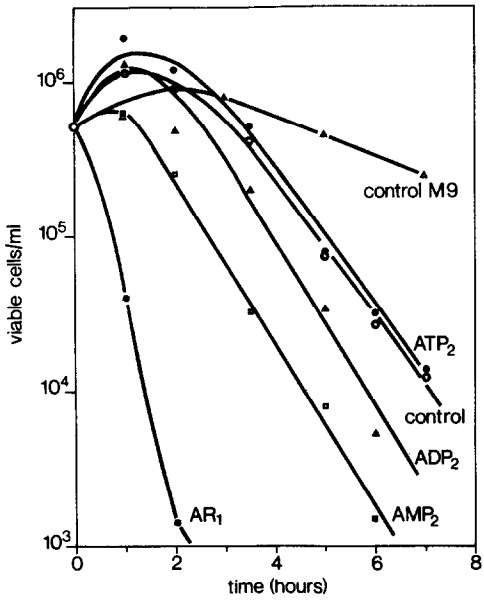

FIG. 5. Effectiveness of adenosine (AR, 1 μg/ml), adenosine-monophosphate (AMP, 2 μg/ml), ADP (2 μg/ml) and ATP (2 μg/ml) in supporting a bactericidal effect of TM (2 μg/ml) in minimal salts medium M9, supplemented by methionine, glycine, serine (30 μg/ml each = control). For comparison, the effect of TM (2 μg/ml) in plain M9 (= control M9) on *E. coli* B is also shown.

period, depending on the TdR concentration. Besides the exhaustion of limiting TdR, the cleavage of TdR by thymidine phosphorylase[16] may be responsible for the onset of cell death. This may be especially true for higher TdR concentrations. In contrast to the immediate action of TdR, even high concentrations of T do not initially prevent cell death, but result in a lower, concentration-dependent number of killed cells. After about 2 hr cell inactivation stops and growth is even resumed.

The composition of biological fluids and drug action. It has been shown recently that an optimal bactericidal effect of antifolates is obtained in media containing amino acids, a purine source, but no T or TdR.[9,11,13,17] These requirements seem to be fulfilled in blood and urine. From the reversal experiments with TdR it can be concluded that TdR concentrations are extremely low and well below 0·05 μg/ml. This will be the subject of a subsequent paper.

Biological fluids contain all amino acids and purine sources.[18] An attempt was made to find out whether the available concentrations of purine sources and amino acids, especially glycine and methionine, were sufficient for optimal drug action. The addition of methionine and glycine to blood and urine did not enhance the action of TM, nor did the addition of purines or their nucleosides such as adenine or inosine. Since some of the purines may be present as nucleotides, the ability of adenosine and its nucleotides to support a bactericidal action of TM was tested (Fig. 5).

From Fig. 5 it can be seen that a concentration of 1 μg/ml of adenosine is sufficient to enhance considerably the TM-action in minimal medium containing methionine, glycine and serine. Lower adenosine concentrations are less effective. The inactivation supporting properties decrease from adenosine to ADP and are absent in ATP, probably owing to restricted uptake. Purines such as caffeine and uric acid were

without effect. The provision of exogenous purine sources, which is obviously met by rather low concentrations therefore seems to be sufficient for an optimal bactericidal drug action in blood and urine.

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