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COMMENTARY

THE BACTERIOSTATIC MECHANISMS OF SULFONAMIDO-TRIMETHOPRIM COMBINATIONS

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In the broadest sense, the bacteriostatic action of sulfonamido compounds (SA) includes those processes which influence their uptake, metabolism and excretion [1–8, etc]. It is of major interest to learn more about the physico–chemical mechamisms of their molecular rearrangment (conformer free energy, quanto–chemical parameters of diverse derivatives, etc) in physiological conditions, as only certain species of these compounds have proven capable of intracellular bacteriostatic activity [3–14, 17, 18, 24, etc].

After SA administration and prior to its binding to the specific bacterial receptors, a significant reduction in the concentration of SA available for binding occurs. SA trapped, altered or excreted by either the host of the invading micro-organism [1–7, 9, 11–13, 15–18, 23] in any way which can not be related to the bacterial 5,6,7,8-tetrahydrofolate (THFA) synthesis is lost for bacteriostasis. Long acting SA is distinct in that its metabolism and rate of release from the plasma protein complexes has been carefully tested to ensure its continued availability for lethal synthesis prior to its introduction into chemotherapeutics [5, 13, 15, 16, 19–21, etc].

Recent recognition of potentiated synergism between SA and 2,4-diaminopyrimidines (Trimethoprim, TMP, etc.) has prompted a reduction in the number of these compounds in clinical use [22, 25–39]. During the past decade, much effort has been extended in order to equilibrate the specific behavior of SA and TMP from the pharmacokinetic and specific antimicrobial points of view. This was also necessitated by the discovery of a host based TMP metabolism [34, 40–43], and of a gradually increasing bacterial resistance [44–49]. It should be noted however, that many authors have reported that SA-TMP conbinations seem to be quite efficient in clinical use.

Advances in our knowledge of the specific receptor systems of *de novo* THFA synthesizing bacteria [50–54] have expanded our understanding of the bacteriostatic/bacteriocidic actions of SA and SA-TMP combinations. The mechanism of SA and TMP actions can be analyzed in both biochemical and quanto–chemical terms [73]. These results suggest the existence of a THFA-multienzyme complex (MEC), containing 2 composite active sites (CAS I and II), into which would fit one molecule each of SA and TMP, as the minimum inhibitory unit. This is the case during bacteriostasis. The only known target of SA action is the THFA-MEC [50–54, 70]. As for TMP, 7,8-dihydrofolate reductase (DHFR), as well as the enzymes of the 7,8-dihydro-6-hydroxymethyl pteridine (Pter) biosynthetic pathway have been identified as specific targets [55]. In addition, DHFR has been implicated as a component of THFA-MEC.

This review will focus itself, therefore, on the biochemical mechanisms of inhibition of THFA-biosynthesis by SA and TMP at the THFA-MEC level.

Formation of lethal products during 7,8-dihydrofolate (DHFA) biosynthesis in the presence of SA

Synthesis, *in vitro*, of 7,8-dihydropteroate (DHPA) from *p*-aminobenzoate (PABA) and Pter, in the presence of ATP, Mg^{2+} and bacterial cell free extracts, was inhibited by various SA compounds [50, 56–62]. Interestingly, the ability of SA to inhibit DHPA synthesis did not parallel inhibition of cell growth. This is attributed to differences in the ability of various SA compounds to penetrate the bacterial cells. It was found though, that each SA was a more potent inhibitor of DHPA-synthesis than of bacterial cell growth.

The idea that SA itself undergoes enzymatic condensation with 7,8-dihydro-6-pyrophosphoryl hydroxymethyl pteridine (Pter-PP) results from experiments in which ^{35}S-labeled sulfanilamide or sulfathiazole was preincubated with pteridine compounds in the cell free extract prior to the addition of PABA. At low pteridine concentrations, there is more inhibition by SA than when substrates and inhibitors are simultaneously combined. At high pteridine concentrations, preincubation resulted in much less inhibition. Subsequent introduction of PABA into the reaction mixtures resulted, in the former case, in the formation of very little DHPA. In the latter case, DHPA synthesis was hardly influenced by the low SA concentration.

The formation of SA containing folate analogues in E. coli has been shown directly using ^{35}S-sulfamethoxazole both *in vitro* and *in vivo* [51].

SA incorporation into folate-like analogues is now well established. On the basis of growth inhibition studies [63], it appears that pteridylmethylaminobenzene sulfamide and pteridylmethylaminobenzenesulfamoylglutamate derivatives so formed inhibit the conversion of PABA, L-glutamate (L-Glu), and Pter to THFA, and metabolic reactions involving the latter in the form of different coenzymes.

It was recognized at the beginning of the SA era that its mode of action was as a competitive inhibitor of PABA [64, 71, 72]. Further elucidation of the molecular mechanism has shown that this is not just a simple, reversible competition for the enzymes' active sites, but rather a more complex, combined inhibition, at the beginning of the enzymatic reaction [65]. Firstly, there is the pure competition for the active

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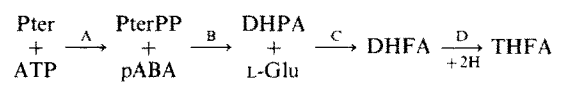
Fig. 1. A non-integrated representation of THFA biogenesis.



A: DHPK; B: DHPA synthetase; C: DHFA synthetase; D: DHFR; Abbreviations are partly introduced in the text.

Fig. 1. Simple representation of THFA biosynthesis as derived from *in vitro* studies with crude and partially purified enzymes. (A) 7,8-dihydro-6-hydroxymethylpteridine: ATP pyrophosphate kinase (HDPK); (B) 7,8-dihydropteroate synthetase (DHPAS); (C) 7.8-dihydrofolate synthetase (DHFAS); (D) 5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase (E.C. 1.5.1.3.) (DHFR).

sites by SA and PABA. Secondly, when active sites are saturated with SA and PABA molecules, both compounds are to be regarded as substrates. Saturation of PABA-binding sites with SA will take place according to its relative momentary concentration to PABA, and relative affinity difference between the two competing compounds. When covalent coupling of pteridine derivatives and SA occurs together in a competitive irreversible inhibition reaction, the concentration of the pteridine compound available for THFA biosynthesis is reduced. This process gradually mounts to the stasis, or death of the cells. One should not ignore the necessary irreversible production of entropy that is associated with the fact that the bacterium cells must function for a time to continuously supply energy for the above synthetic reactions.

Complex nature of the enzymes of DHFA biosynthesis

DHFA assembly includes a series of specific enzymatic reactions. Many excellent reviews have been published on this subject [66, 67], as well as on the MEC of DHFA/THFA assembly [52–54]. The advantages to the cell of such a superorganized system have only recently been assessed [68, 69]. In DHFA/THFA biosynthesis, recognition of the enzyme's superorganization brought the realization that further elucidation of the structure, function and enzymatic action mechanism, specific SA and TMP receptors, etc would be of value in the formulation of new antifolate chemotherapeutica.

Figure 1 summarizes the biogenesis of THFA. This is a simplified representation, showing only DHFA assembly from its components, and the subsequent reduction to THFA.

Evidence has been accumulating from various sources showing that heterologous protein associations among THFA biosynthetic enzymes can occur *in vitro*. This supports the idea of a similar condition ocurring *in vivo*. The present status of THFA biosynthetic enzyme aggregation capability has been reviewed in a series of articles by this author [53, 54]. An important facet of the results is the subunit structuralization of the aforementioned enzymes. Separation of specific and stoichiometric PABA-, L-Glu-, Pter-, and 2 different ATP-binding subunits, as well as DHFR were observed in the final step of enzyme purification after treatment by 4 M guanidine. HCl and 0.05 M EDTA. From their respective subunits, individual enzymes (A–C) were reconstructed in an equimolar fashion. It was assumed that these subunits, arranged in a given manner, represent the enzymes of THFA biosynthesis. During preparation, the physiologic THFA-MEC was subject to dissociation. As a result, individual subunits, and partial heterologous associates, including enzymes A, B, and C, appeared in the extracts. It is also believed that the THFA-MEC aggregates contain more than one subunit each. Thus the "THFA-MEC" [53] is now regarded [54] as a monomer of the MEC with an apparent molecular weight of about 150,000 daltons. According to a speculative pentameric model, the molecular weight can range up to 500,000 daltons. Table 1 summarizes the subunit structuralization of THFA biosynthetic enzymes. These are depicted as THFA-MEC dissociation products by Fig. 2. In addition, this figure shows two possible composite active sites (CAS) in which primary PABA/DHFA and Pter activation can occur without formal movement of the substrates from enzyme to enzyme. Motion is restricted to those specific oscillations of the substrate molecules necessary to link chemical reactions at different sites to transmit free energy which must be high grade macroerg acid anhydride of P ~ P or −CO ~ AMP. This is followed by DHFA synthesis randomly [53, 62] from the activated components, and finally by reduction to THFA. Some of the free energy of interaction between the subunits and the first ligands will likely distort the protein structure towards a conformation that favors the binding of subsequent ligands of other subunits [74]. This model has not yet been elucidated as to free energy migration, involving not only positive cooperation of subunit conformations, but also electrostatic interactions, solvent (water) and chemical reaction site considerations.

Table 1. Subunit structuralization of partial heterologous enzyme associates of the THFA-MEC

Subunit	Mol. wt (daltons)	Enzyme to which the subunit is a component*	Reference
pABA-binding	15,000	DHPAS I, −II; DHFAS I, −II; pABGAS	54
Pter-derivative-binding	40,000	DHPK; DHPAS I, −II; DHFAS I, −II	54
L-Glu-binding	35,000	DHFAS I; pABGAS	54
ATP-binding I	15,000	DHPK; DHPAS I	54
ATP-binding II	28,000	DHFAS I; pABGAS	54

* Abbreviations are introduced in the text and at Figs. 1 and 2.

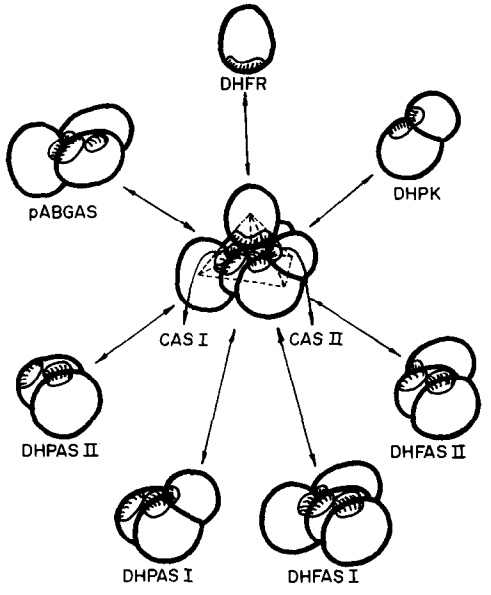


Fig. 2. Proposed subunit structuralization of the E. coli B THFA-MEC. Individual enzymes are shown as dissociation products of the MEC. Location of composite active sites (CAS I, and CAS II) are indicated by the shaded areas in both the MEC and the enzymes.

It has been calculated that there are only about 300 THFA-MEC aggregates in each *E. coli* B cell. Kinetic studies with both native and reconstituted THFA-MEC may reveal a specific substrate–product flux kinetic across the MEC [69]. THFA-MEC associations with small particles (i.e. mitochondria) has not yet been ruled out. In other words, we are only now beginning to understand THFA biosynthesis as an integrated process.

Mechanism of inhibition of DHFA biosynthesis by SA and TMP in the THFA-MEC

There are two composite active sites in the MEC.

CAS-I is composed of the amino acid residues corresponding to the following ternary associated subunits: PABA binding subunit + ATP binding subunit II (PABA: ATP adenylate kinase) + L-Glu binding subunit. The ternary associate has already been termed the *p*-aminobenzoylglutamate synthetase system (pABGAS) [54].

On the surface of the PABA binding subunit, there should be + and − net charges, about 6.7 Å apart for PABA or SA fixation. Amino acid residues, reacting with the negative charge of the benzene ring (the result of its characteristic π-electron delocalization), should be located between the charges.

The L-Glu binding subunit has + charges for binding L-Glu by its −COOH units. These are arranged in such a way that the α-amino group is turned toward CAS I. On the surface of the DHFR molecule, looking toward the L-Glu binding subunit, the mirror image of the above charge distribution must be present. This is strongly suggested by experiments testing for *p*-aminobenzoylglutamate (PABGA) binding site region of this enzyme with PABGA analogues [66].

On the intersubunit surfaces of CAS I, amino acid residues, suitable for quaternary structure formation, and Mg^{2+} ions ensure stability. The importance of Mg^{2+} ions for stability has already been shown by experiments carried out in the presence of chelating agents, such as EDTA [54].

The existence of CAS I is also supported by the fact that PABA ~ AMP (DHFA ~ AMP), although it is an obligatory intermediate and can't be detected in the system because of the channeling effect. Thus, a mechanism is established (Fig. 3) so that the subunit collaboration on adenylation, as an activation of PABA, is followed by a minimum movement (push) of its −COOH towards the attracting (pull) primary amino group of the L-Glu molecule. This kind of push–pull mechanism (Fig. 4) can only be proven by active center analysis, but it is strongly supported by the inseparability of the elementary reactions. In the absence of L-Glu, the substrates PABA and ATP can only saturate the active sites of the corresponding enzymes, because formation and accumulation of PABA ~ AMP cannot be detected in the medium.

During activation with ATP, and reaction with L-Glu, the carboxylic group of PABA, is essentially immobile, except for the aforementioned oscillations between the active sites of polypeptides forming CAS I and those of DHFR.

The amino group of PABGA is less basic due to an intramolecular, mesomeric rearrangement of its electron system. As PABA ~ AMP moves toward the amino group of L-Glu during the reaction which forms PABGA, the mesomeric rearrangement of PABGA takes place. The strong nucleophillic attraction (pull-back) of the pyrophosphoryl group of Pter-PP prevails and the reaction between the primary amino group of PABGA and the primary alcoholic ester-hydroxyl of Pter-PP can take place (Fig. 4). At the same time, the PABGA portion of the newly formed DHFA molecule can further oscillate between the specific binding sites of CAS I and DHFR.

CAS II is composed of the active sites and the accessory binding regions (hydrophobic) of the following subunits: 7,8-dihydro-6-hydroxymethylpteridine: ATP pyrophosphokinase (DHPK, also known as ATP binding subunit I) + Pter derivative binding subunit + DHFR.

Pter binds up in the active site of Pter binding subunit, as well as the TMP inhibitor, in such a manner that only the primary 6-hydroxyl group spreads to the active center of DHPK. Phosphorylation is followed by a 90° flipping over (Fig. 3), accompanied by the simultaneous elimination of 5'-AMP from the MEC. DHFA is formed by the active collision with the primary amino group at PABGA. The appearance of the DHFA molecule is a signal allowing the double bond between N5 and C6 to be set close to the active site of DHFR by a slight rotation of the plane of Pter around the imaginary diagonal between N3 and C7.

The linkage between CAS I and CAS II is mediated mainly through the PABA binding subunit, but is supported by a loose contact between DHFR and the corresponding complementary CAS.

TMP has a remarkable affinity for Pter binding subunit. Therefore, A, B, and C enzymes can partially be inhibited by this substance, although DHFR is its

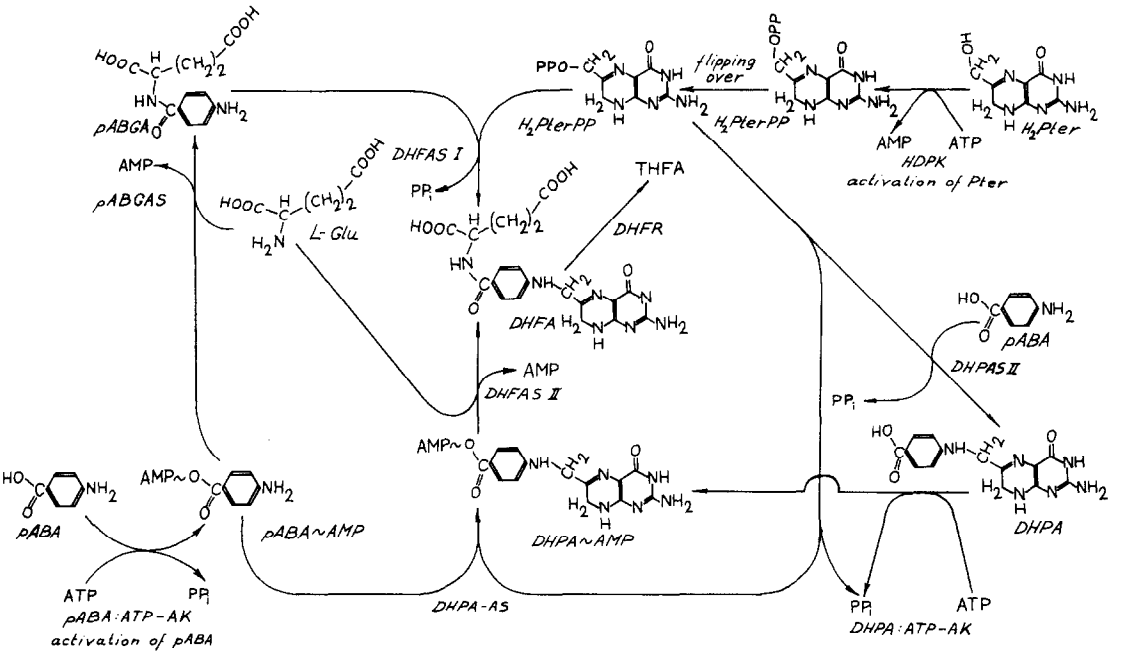


Fig. 3. Integrated pathway of THFA biosynthesis. Three possible paths of THFA synthesis can be seen on the figure: (a) condensation of Pter-PP and pABGA, following synthesis of the latter from pABA ~ AMP and L-Glu; (b) direct formation of DHPA ~ AMP is followed by coupling of the active compound with L-Glu, or (c) DHPA can form primarily, and activation of it will be the next step.

principal target enzyme [54]. Our preliminary results [55] showed partial inhibition of GTP-cyclohydrolase, 7,8-dihydroneopterintriphosphate pyrophosphorylase and neopterin aldolase with TMP, because, in a portion of their active sites, the same Pter ring system is bound with varied affinity. These findings can serve as the basis for speculation as to the true nature of the SA-TMP synergism. It seems likely that a series of partial inhibitions of the enzymes involved in the Pter precursors' biosynthesis and THFA assembly amplification of TMP inhibition can occur. In the integrated system, the possibility of a cascade inhibition mechanism is given *ab ovo*, and this is extended to the related Pter biosynthesizing system. No data has been found, however, for SA, that would support the idea of an extended bacterial inhibition mechanism similar to that of TMP. A number of valuable articles and reviews give details of DHFR inhibition by TMP and related compounds [25–31].

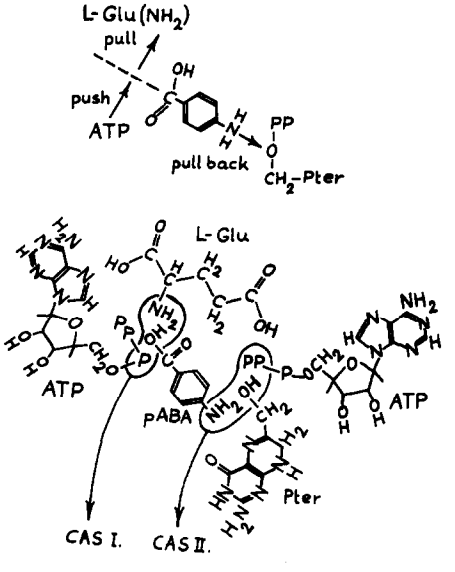


Fig. 4. Top view of the mechanism of THFA-assembly in the reaction pool. (below), and the push-pull mechanism (above).

Lethal blockade of vital bacterial metabolic processes at the level of THFA biosynthesis, and new principles of designing anti-folate drugs

THFA plays an important role in a great variety of vital metabolic processes, such as purine ring synthesis, histidine and purine degradation, methionine ($-CH_3$) synthesis, thymidylic acid synthesis which is related to DNA synthesis, essential in cell division.

The dose reduction made possible by SA-TMP combinations has reduced to a minumum many of the side effects associated with sulfa drugs. Despite this reduction in the *in vivo* concentration of these chemotherapeutic agents, one can see not only bacteriostatic, but bacteriocidic effects. These combinations have been successfully extended to very severe cases of brucellosis [75, 76], salmonellosis [77, 78], typhoid, paratyphoid B [79, 80], shigellosis [81], and, to a limited extent to pseudomonas pyocianea infection as well as to the control of proteus mirabilis septicemia [44]. Parasites, widely different from a microbiological point of view, can be attacked with SA-TMP combinations. The bulk of information which has become available concerning mode of action of SA-es and TMP on sensitive micro-

organisms, permits us to draw the cautious conclusion that the mechanisms involved in SA-TMP combined action essentially do not show a great variety of alterations. Therefore, it can be expected that enzymes producing THFA by the same types of biochemical reactions are made up to the same types of subunits associated in the same way to build up the same type of MEC-es in all *de novo* THFA biosynthesizing SA-TMP sensitive microorganisms. Such structures do not exist in the cells of host organisms.

In designing new anti-metabolites of THFA biosynthesis, oriented and covalent linking of SA and TMP derivatives seems to be promising in producing competitors for the physiological substrates. Chemical linkage of SA and TMP derivatives provides the 1:1 momentary and steady molar ratio of the inhibitors at the specific and lethal microbial active sites. In the case of active compounds, the problem of different pharmacokinetics of SA-es and TMP is ruled out in this way. Another advantage of the compounds could be that by hiding of SA and TMP parts of the molecule in new chemical structures, specific SA and TMP resistance factors may be inert towards them. Unexpected actions of these new compounds are, however, likely to occur.

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