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Mechanism of Action of the Folate Blocker Diaminodiphenylsulfone (dapsone, DDS) Studied in *E. coli* Cell-Free Enzyme Extracts in Comparison to Sulfonamides (SA)[1]

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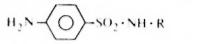
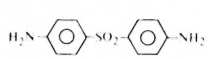
The exceptionally high antibacterial activity of DDS against *M. leprae* ([12, 18]) leads to the question of the underlying molecular mechanism. The special situation in leprosy, i.e., the lack of an *in vitro* system to cultivate *M. leprae*, forced us to study the mechanism of action and structure-activity relations of sulfones in model strains in order to search for the reasons for their extremely high activity against *M. leprae* (0.0016 μmol/l in the mouse foot pad ([11, 12, 18, 28])). In studies with other bacterial strains, it has been found that sulfonamides (SA) (I) and sulfones (DDS) (II) act as "folate blockers."



I



II

Recognition of the importance of p-aminobenzoic acid (PABA) in folic acid metabolism and the ability of certain folate derivatives and/or products to reverse the effect of SA or sulfones has led to a general theory of SA and DDS action. In principle, this theory states that SA and DDS are competitive inhibitors of the enzymatic incorporation of PABA in folic acid synthesis ([20]). The sequential pathway of folic acid synthesis has been evaluated by Jaenicke and Chan ([9]), Brown ([4, 5]), Shiota, *et al.* ([29]), and Ortiz and Hotchkiss ([16]) and is given in Fig. 1. It has also been shown that bacterial- ([16, 31]), plant- ([8, 15]), and plasmodial- ([6]) cell-free folate synthesizing extracts are inhibited by SA.

MATERIALS AND METHODS

Determination of DDS and sulfonamide inhibitory activities i_{50} and k_{50} in a cell-free system. The ratio of the amount of folate produced in the reaction mixtures (reactants and concentrations as described by Miller, *et al.* ([14])) containing DDS or sulfonamides respectively to that produced in controls was used as fractional activity a. The concentration of inhibitors causing a 50% inhibition of folate synthesis was obtained by plotting $i = 1 - a$ vs. the logarithm of the inhibitor concentration (DDS or SA). In kinetic experiments the rate of folate synthesis was evaluated graphically from the initially linear portions of plots of the amount of folate synthesized vs. time. The concentration of DDS or SA causing a 50% inhibition in the rate of folate synthesis was evaluated from plots of $1/(k_0 - k_i)$ vs. the reciprocal of the inhibitor concentration where k_0 and k_i are the rate constants for folate synthesis in the absence and presence of inhibitor. For a detailed description, see Miller, *et al.* ([14]).

Assay of folate growth equivalents. The methods have already been described ([14]).

Minimum inhibitory concentrations (MIC). The methods for MIC determination have been previously described ([10, 23, 24]).

QSAR analysis. Statistical analysis was performed with a Wang computer 700B or 2000 VP. For all regression equations the number of data points or number of compounds used is n; the correlation coefficient r, the standard deviation s, and the F-test are given.

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Pteridine derivatives. The pteridine analog of DDS 6-(N-4,4'-diaminodiphenylsulfonyl) methyl-pteridine has been synthesized in accordance with the description already published for the SA analog N'-d-(5-methylisoxazolyl)-N^4-(6-pteridinylmethyl) sulfanilamide ([3]).

nmr (DMSO, 90 MH_z) δ 8.72 (S 1H)
AA'BB' centered at 6.65 and 7.53 (J = 8H_z, 8) 4.52 (S 2H)
uv (0.1 N NaOH) 365 nm (ϵ = 6.84), 300 nm (ϵ = 19.05), 225 nm (ϵ = 26.38)

Enzymatic reaction mixtures for the determination of reaction rates for DDS and SA incorporation into a dihydrofolic acid analog. The reactants and concentrations were as described previously ([2]) with the exception that PABA derivatives were replaced by DDS or SA.

Bacterial growth kinetics. *E. coli* (mutaflor) maintained on agar slants was used as the test organism. The culture broth was dextrose-salts-casamino acids (vitamin-free), which has been described by Anton ([1]). Trimethoprim (TMP) was supplied by Deutsche Wellcome GmbH, Grossburgwedel; PABA and DDS were from commercial sources.

Growth. A broth culture was inoculated from an agar culture and allowed to grow for 12–16 hr. All cultures were grown at 37°C. A dilution of this culture—the preliminary culture—was allowed to grow into the logarithmic phase. When a concentration of about 10^7–10^8 organisms/ml was attained, this culture was used to prepare experimental probes (10^4 organisms/ml).

Total count (Coulter Counter). Samples of the experimental cultures were diluted with particle free saline (0.85%)-formaldehyde (0.2%) solutions so that a count of 1000–20,000 organisms was obtained. Diluted samples were counted with a Coulter Counter model "ZB" equipped with a 30 μm orifice. Counts per 50 μl were obtained. Instrument settings were: 1/aperture current 1; 1/amplification ½; matching switch 40 K; gain 10; lower threshold 7 and upper threshold maximum ([26, 27]). Time of drug addition was as indicated.

RESULTS AND DISCUSSION

Determination of cell-free activity (i_{50}) of SA and DDS. Brown ([5]) has determined inhibitory activities of several SA in such a cell-free folate synthesizing system. He reported that these activities in general are proportional to the antibacterial activities (minimal inhibitory concentrations or MIC) of these compounds. The relatively small number and the heterogeneity of the studied compounds, however, have limited utility for detailed quantitative structure-activity relationships (QSAR). Similarly, the data have limited utility in assessing the role of permeability as it relates to antibacterial activity.

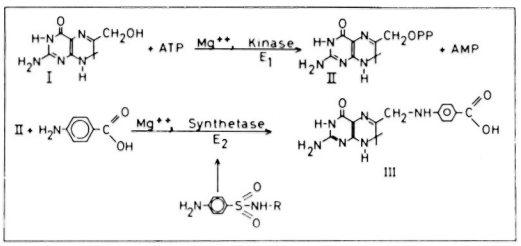
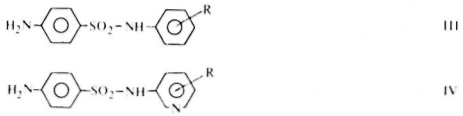


FIG. 1. Reaction scheme of 7,8-dihydropteroic acid (III) biosynthesis.

To gain more information about structural requirements and the physicochemical forces which are directly related to the enzyme-SA or enzyme-DDS interaction and to find an explanation for the observed optimum in whole cell activity (MIC), the inhibitory activity of DDS and of a closely homologous series of SA[14] have been studied in our laboratories. Results of some typical (single point) experiments conducted to determine the inhibitory activities of several N^1-phenyl (III) and N^1-pyridyl (IV) sulfanilamides and DDS (II) are given in Figs. 2 and 3.



They show a plot of fractional inhibition of folate synthesis against the inhibitory concentration used, i.e., dose response curves. The amount of folate synthesized was determined after a fixed time interval (3 hr). From such plots the SA or DDS concentration was determined which caused 50% inhibition of folate synthesis (i_{50}). The tested SA ($\approx$50 in total) show a wide range in physicochemical properties (e.g., lipophilicity π, log P), electron distribution (pKa), and steric effects (E_s) and exhibit an ap-

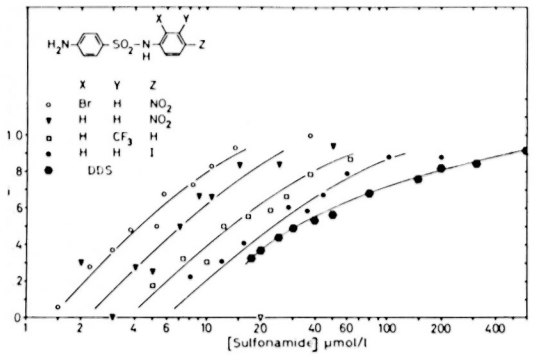


FIG. 2. Determination of the inhibitory activity of several N¹-phenyl sulfonamides and of DDS. Plots of inhibition (i) of cell-free folate synthesis versus log of the SA or sulfone concentration were used to determine i_{50} values (after 3 hr).

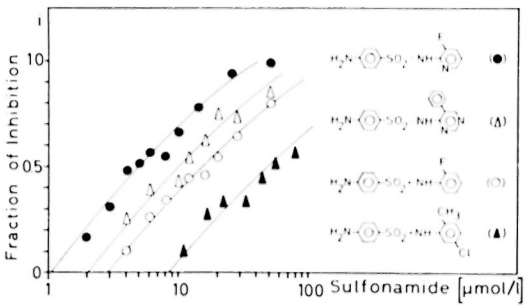


FIG. 3. Determination of the inhibitory activity of several N¹-pyridyl sulfonamides. Plots of inhibition (i) of cell-free folate synthesis versus log of the SA were used to determine i_{50} values (after 3 hr). [19]

proximately 25-fold variation in inhibitory activity in the cell-free system. It is interesting to note that the shapes of the dose response curves for all highly active SA are identical whereas the shape for the DDS curve is different (Fig. 2). For SA the concentration for the half maximum inhibitory effect is almost half the concentration for the maximum effect (total inhibition) whereas for DDS the concentration needed for total inhibition is almost 12 times higher than for the half maximum effect. It has been stated that the ratio of the drug concentration causing 90% inhibition over that causing 10% inhibition (I_{90}/I_{10}) for competitive inhibition is usually around 100 while the ratio for inhibition by reactions with substrate is usually between 5 and 25 [30]. For highly active SA we observe a ratio of approximately 10 and with DDS a ratio of approximately 70. The inhibitory potency of DDS in *E. coli* cell-free systems is approximately 20-fold less than that of the most active SA. This is in contrast to the statement of Maren [13], who found the inhibitory activity of DDS to be comparable to that of sulfapyrimidine. Similar activities of DDS have been found in cell-free systems obtained from *Plasmodium berghei* (i_{50} = 22 µmol [21]), compared to a cell-free system of *E. coli* (i_{50} = 35 µmol) (see also Ferone [6]).

Comparison of whole cell inhibitory activities (MIC, *E. coli*) and cell-free system inhibitory activities (i_{50}). If the inhibitory activity in whole cell systems (MIC) is plotted in a double logarithmic system against the activity we have found in the cell-free system (i_{50}) [14], a linear relationship is obtained (Fig. 4). It is apparent that with the exception of 4 compounds (43, 44, 45 and 47), the results with all SA and dapsone can be described by an equation for a straight line:

	n	r	s	F	
$\log i_{50} = 0.64 \log \text{MIC} + 0.75$	22	0.95	0.13	187	(1)

This relationship indicates that the differences in MIC values are paralleled by differences in cell-free activities. Therefore, these differences cannot be attributed to permeability factors but must be associated with the reactions of the SA and sulfones with the enzyme proteins of *E. coli*. It seems therefore most likely that permeability factors do not contribute substantially to the antibacterial activity of the tested compounds *in vitro*. The results are good evidence that the rate determining step in both systems is the same and is not the permeation step (associated with the rate constant k_1 in Fig. 5). Other reaction steps which could be rate determining are competition with the natural substrate, PABA, by SA or sulfones for binding sites on the enzyme (associated with the rate constant k_2 in Fig. 5) or the reaction rate of product formation (k_3 in Fig. 5).

QSAR of whole cell and cell-free activities. The observed variance in MIC values has been correlated to and explained by changes in the physicochemical properties of the o-, m-, and p- substituted SA. In this correlation the pKa value was expecially important as an indicator of changes in electron distribution [22]. To account for

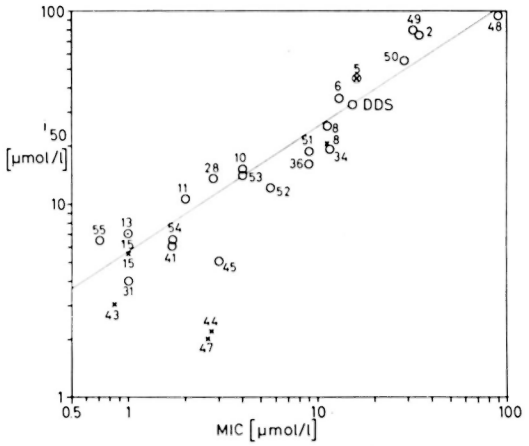


FIG. 4. Relationship of cell-free inhibitory activities (i_{50}) to whole cell inhibitory activities (MIC, *E. coli*). For discussion of deviating compounds 43–45 and 47 see text. ([14]) Numbers designate different compounds.

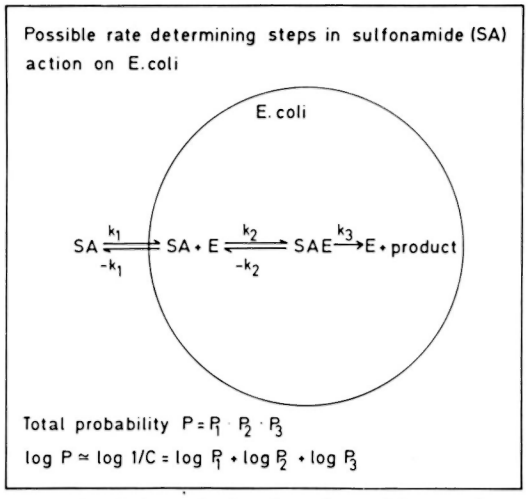


FIG. 5. Schematic drawing of possible rate determining steps in antibacterial action.

a special "steric" effect in case of the o-substituted SA, a dummy parameter, D, has been introduced into the regression equation (2) having the value 1 for o- substituted derivatives and 0 for m- and p-substituted SA. The following equation was obtained:

$$\log \mathrm{MIC}_{o,m,p} = 0.67\mathrm{pKa} - 0.24D - 4.74 \quad \begin{matrix} n & r & s & F \\ 18 & 0.95 & 0.14 & 67 \end{matrix} \qquad (2)$$

A similar analysis to explain the observed variance in cell-free activities (i_{50}) resulted in the following equation:

$$\log i_{50}{}_{o,m,p} = 0.46\mathrm{pKa} - 0.33D - 2.47 \quad \begin{matrix} n & r & s & F \\ 18 & 0.97 & 0.08 & 123 \end{matrix} \qquad (3)$$

The dependencies on structural changes are almost identical in both systems, thus supporting the assumption that the interaction with the enzyme proteins is the rate determining step (associated with the rate constant k_2 in Fig. 5).

Kinetics of inhibition and formation of analogs. The deviation of compounds 43, 44, 45 and 47 in Fig. 4 leads us to an interesting observation. These compounds, which show more potent inhibitory activities in a cell-free system (i_{50}) than in whole cells (MIC) (Fig. 4) but still less than expected according to equation 3, have very low pKa values (≤6.5), i.e., they are more than 90% ionized under the conditions of the experiment. For these compounds the kinetics of inhibition was determined in a cell-free system. An example is given in Fig. 6 for DDS. The observed k_{50} values (concentration

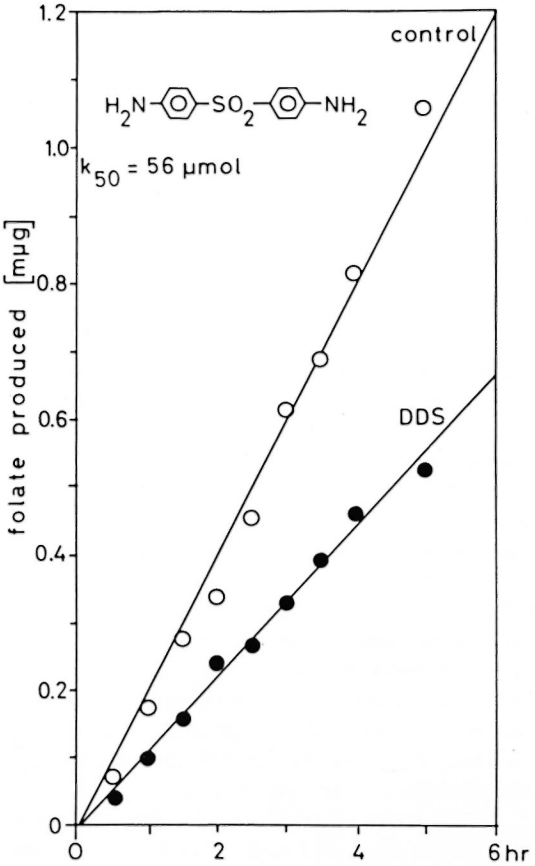


FIG. 6. Determination of inhibitory activity of DDS by the kinetic method.

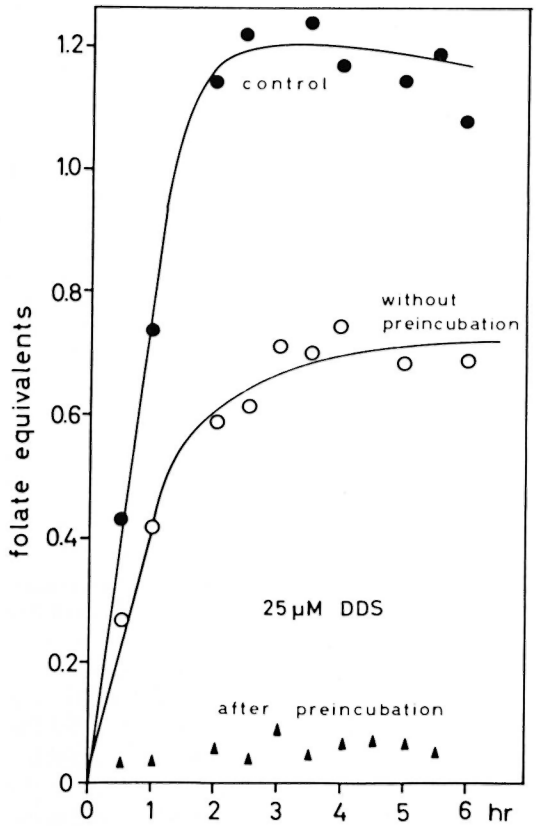


FIG. 7. Kinetics of inhibition of folate synthesis by DDS (25 μM) with (▲) and without (○) preincubation. In the case of preincubation 25 μM DDS were preincubated for 3 hr with 7,8-dihydro-6-hydroxymethylpterin (32 μM), and PABA (20 μM) was subsequently added.

necessary to reduce the rate of dihydrofolic acid synthesis to one half) are in agreement with the observed i_{50} values (concentration needed to reduce the amount of folate produced after 3 hr to one half). For the very active SA, however, the k_{50} was much smaller than the i_{50} for the first 2–3 hr interval. Later, the rate of folate synthesis returned to the rate of the control probe. This behavior indicates a "use up" of inhibitor in a reaction with the substrate dihydropteridine alcohol ([14]). In the case of SA this has been demonstrated, and the dihydropteroic acid analog has been isolated and identified from cell-free reaction systems and from whole cells ([3, 25]).

Studies performed in our laboratories revealed that in the case of DDS, an analog of dihydropteroic acid is formed. If in a cell-free folate synthesizing enzyme system

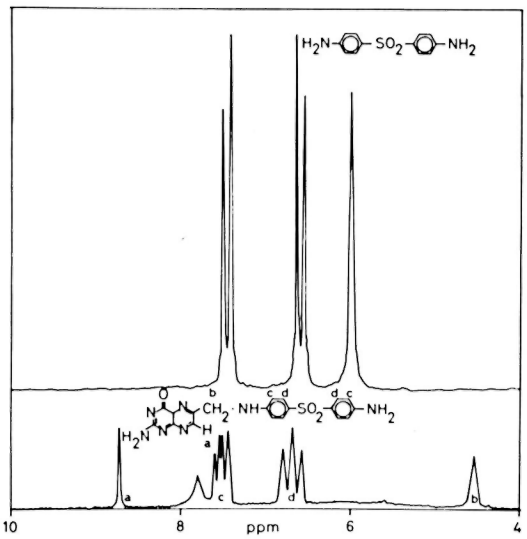
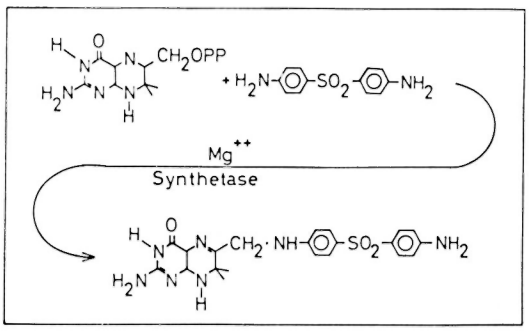


FIG. 8. NMR spectra of DDS and its dihydropterinyl analog (solvent DMSO).

the substrate dihydropteridine alcohol pyrophosphate is incubated together with DDS in the absence of competing PABA, no production of dihydropteroic acid occurs (Fig. 7) when PABA is subsequently added because all precursor dihydropteridine alcohol pyrophosphate has reacted with DDS to form the analog. The dihydropteroic acid analog—where the PABA moiety has been replaced by DDS—has been synthesized and the structure identified by NMR spectroscopy (Fig. 8). The biochemically synthesized product is identical with the synthetic product.



To examine if the rate determining step in inhibition of folate synthesis by SA and sulfones is the competition for binding sites or the rate of formation of the dihydropteroic acid analog, associated with the rate constant k_3 in Fig. 5, the kinetics of analog formation was studied in the absence of PABA. The decrease in substrate concen-

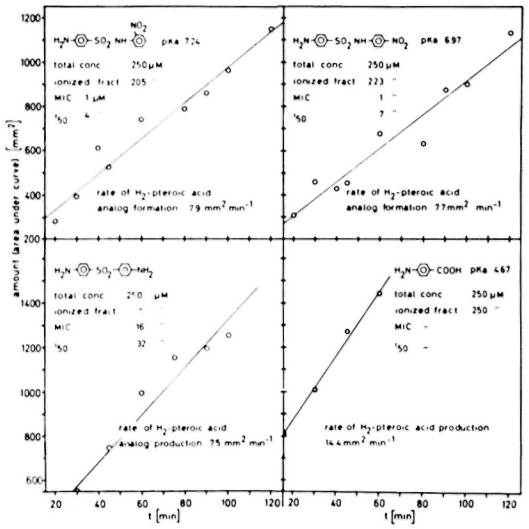


FIG. 9. Rate of formation of "folate analogs" by various SA and DDS in absence of PABA (cell-free folate synthesizing enzyme system *E. coli*).

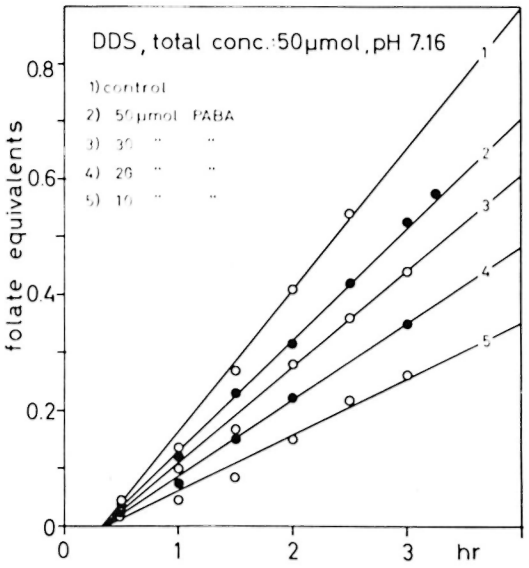


FIG. 10. Reversal of DDS inhibited folate synthesis by increasing concentrations of PABA (cell-free folate synthesizing system *E. coli*).

tration (dihydropteridine alcohol) and the formation (increase) of analog has been followed by quantitative thin layer chromatography ([2]). The results are given in Table 1 and Fig. 9. All SA derivatives studied and DDS show the same rate of analog formation despite the large variance in their inhibitory power (only PABA shows a faster rate for its incorporation into dihydropteroic acid). Therefore the rate determining step in folate inhibition is not the rate of formation of the dihydropteroic acid analogs but the different affinity of the inhibitory SA and sulfones to the enzyme dihydropteroic acid synthetase compared to the affinity of the natural metabolite PABA expressed by the Michaelis-Menten constant K_m. The molar concentration of PABA needed to reduce the inhibitory effect of DDS (Fig. 10) and of various SA (at constant concentration of the ionized form) to

TABLE 1.

Rate of H_2-pteroic acid analog formation by various N^1-phenylsulfanilamides (SA) in a cell-free system in the absence of PABA at pH 7.71, 37 °C

H_2N-⬡-SO_2·NH-⬡-R	SA-conc. [µM] total	ion. fract.	MIC [µM]	i_{50} [µM]	rate [$mm^2\ min^{-1}$]	pKa	π	D
4-CH_3	5239	223	21.8	–	6.50	9.25	0.44	0
H	2840	223	16.0	45.0	7.40	8.97	0	0
4-Cl	1242	223	13.0	35.0	7.58	8.56	0.83	0
4-Cl	273	49	13.0	35.0	7.90	8.56	0.83	0
2-Cl	991	223	2.8	13.5	6.35	8.18	0.37	1
2-Cl	341	117	2.8	13.5	6.65	8.18	0.37	1
2-NO_2	250	205	1.0	4.0	7.90	7.24	0.12	1
4-NO_2	250	223	1.0	7.0	7.70	6.97	0.59	0
PABA	250	250	–	–	14.00	4.67	–	–
DDS	250	0	16.0	32.0	7.50	–	log P 0.9	0

TABLE 2.

Competitive activity of PABA (K_{PABA}) against various N^1-phenylsulfanilamides (SA) at constant concentration of ionized SA at pH 7.71, 37 °C, in a cell-free system (PABA concentration range 10 - 50 µM)

H_2N-⬡-SO_2·NH-⬡-R	SA-conc. [µM] total	SA-conc. [µM] ion. fract.	MIC [µM]	i_{50} [µM]	K_{PABA} [µM]	pKa	π	D
4-OCH_3	436.7	10	34.5	75.0	59	9.34	-0.12	0
H	185.2	10	16.0	45.0	68	8.97	0	0
4-Cl	80.0	10	13.0	35.0	59	8.56	0.83	0
4-J	38.6	10	11.3	25.0	63	8.17	1.12*	0
4-$COCH_3$	16.3	10	2.0	10.5	54	7.52	-0.55*	0
4-NO_2	12.0	10	1.0	7.0	65	6.97	0.59	0
2-Cl	39.4	10	2.8	13.5	93	8.18	0.37	1
2-NO_2	13.4	10	1.0	4.0	100	7.24	0.12	1
DDS	50	0	16.0	35.0	16	-	log P 0.9	0

*literature value

one-half of the effect in the absence of PABA is given in Table 2 and Fig. 10 (cell-free system). It is obvious that the ionized form of SA is a more powerful inhibitor of folate synthesis than dapsone. This is in agreement with the other results obtained with *E. coli*, especially the MIC.

So far, the results of mechanism of action studies of sulfones in a cell-free system and in whole cell systems of *E. coli* and also in plasmodia seem to indicate identical inhibitory behavior compared to SA. Also, in a strain of mycobacteria (*M. kansasii*) where DDS shows a remarkable increase in inhibitory power (MIC 0.3 – 0.5 µg/ml = 1.2 – 2.0 µmol/l), the inhibitory effect can be totally antagonized by PABA (PABA:DDS = 2:1) ([17]).

Bacterial growth kinetic studies. To get further information which might indicate a deviation in the mechanism of action of DDS in comparison to SA—at least to a certain degree—bacterial growth kinetic studies have been performed ([7, 27]). In these studies the rate of growth of bacteria is determined during the exponential growth phase in the absence and presence of inhibitors. The number of organisms in a defined volume of culture medium is counted with a Coulter Counter as a function of time and inhibitor concentration. If the number of bacteria (N) per volume is plotted against time (t) in a semilogarithmic graph, a straight line is obtained:

$$\log N = \log N_0 + \frac{kt}{2.303} \qquad (4)$$

From the slopes of these curves the generation rate constant (k) can be calculated. Figure 11 gives an example of the growth of *E. coli* in the absence and presence of various concentrations of DDS. In agreement with the results in the case of SA ([27]), a lag phase of approximately 5 generation times is observed before onset of inhibition. The lag phase is independent (sampling every 30 min) of the type and concentration of the SA or sulfone used and is due to a pool of dihydrofolate within the bacterial cell. After depletion of this pool, a new steady state growth as a function of inhibitor concentration is obtained. It is interesting to note that a second stronger inhibition phase is observed after a constant time interval if the cultures are treated with DDS. The reason for this is not yet understood. This point will be stressed later.

The relation between concentration and inhibitory effect (decrease in generation rate constant) can be linearized by the fol-

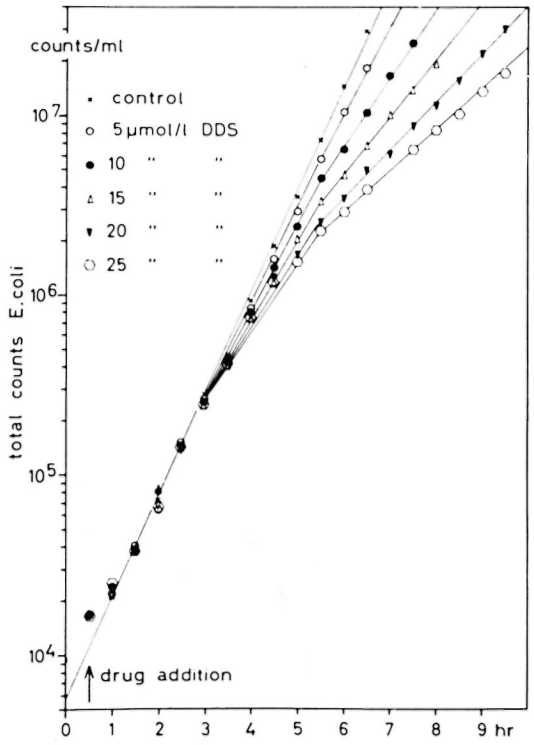


FIG. 11. Typical generation rate curves of *E. coli* (mutaflor) at 37°C in the presence of various concentrations of DDS. The curves, final drug concentrations (µmol/l) and apparent steady state rate constants, $k_{app} \times 10^{-4}$ in sec^{-1} were: (x) control 3.63; (○) 5 DDS, 3.43; (●) 10 DDS, 3.09; (△)15 DDS, 2.8 and for the second inhibition phase: (○) 5 DDS, 3.13; (●) 10 DDS, 2.45; (△) 15 DDS, 1.97; (▼) 20 DDS, 1.71; (◌) 25 DDS, 1.39.

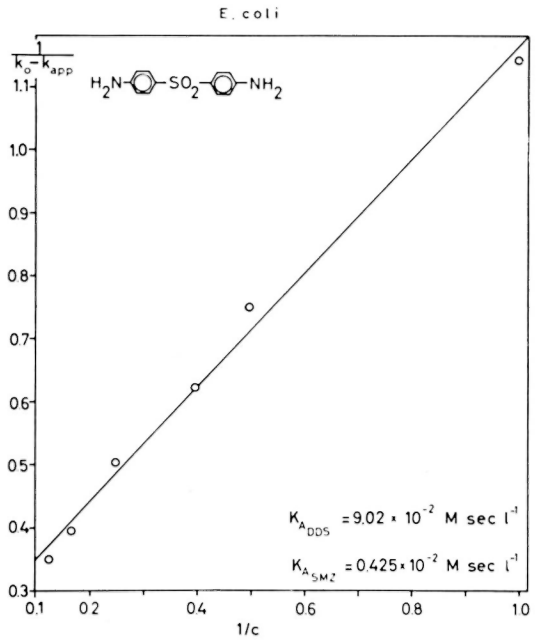


FIG. 12. Example of quantitative relation between apparent *E. coli* growth rate constants k_{app} and DDS concentration (total counts). The curve is plotted in accordance with the equation: $1/(k_0 - k_{app}) = \frac{1}{C} K_A + K_B$.

lowing equation (Lineweaver-Burk-Plot):

$$1/(k_0 - k_{obs}) = \frac{1}{C_{DDS}} K_A + K_B$$

where k_0 is the rate constant of the control culture, k_{obs} the rate constant in the presence of DDS, C_{DDS} is the DDS concentration, K_A the activity constant obtained from the slope of a curve where $1/(k_0 - k_{obs})$ is plotted against 1/C (see Fig. 12), and $K_B = 1/k_0$ for $C \to \infty$. The obtained activity constant for DDS was $K_{A_{DDS}} = 9.02 \times 10^{-2}$ M·sec·l^{-1} and for 3-sulfa-5-methylisoxazole $K_{A_{SMZ}}$ was 0.425×10^{-2} M·sec·l^{-1}. The ratio of the activity constant $K_{A_{SMZ}}$ for these two compounds is the same as the ratio of the MIC values ($MIC_{DDS} = 16$ µmol/l; $MIC_{SMZ} = 0.8$ µmol/l; *E. coli*). Also, the synergistic behavior of DDS in combination with a dihydrofolate inhibitor like trimethoprim (TMP) or pyrimethamine ([26]) is comparable to the synergism observed in the case of SA in combination with TMP (Fig. 13). The mechanism of the synergism of combinations of SA and TMP or DDS and TMP is still not understood. As a hypothesis especially to explain the extraordinarily high antibacterial effect of DDS alone against *M. leprae*, we would propose that the formed DDS-dihydropteroic acid analog might act itself at the dihydrofolate reductase level, thus causing a more than additive (i.e., synergistic) effect to the inhibitory power of dapsone alone.

Poe ([19]) has also made a proposal to explain the synergism observed for combinations of SA and dihydrofolate reductase inhibitors. He assumes that SA and also DDS act not only at the enzyme dihydropteroic acid synthetase (Fig. 1) but also at the dihydrofolate reductase. The published K_i values (see Table 3) for various SA and DDS acting on dihydrofolate reductase show, however, that they are at least two orders of magnitude larger than the inhibition constants observed for inhibition of

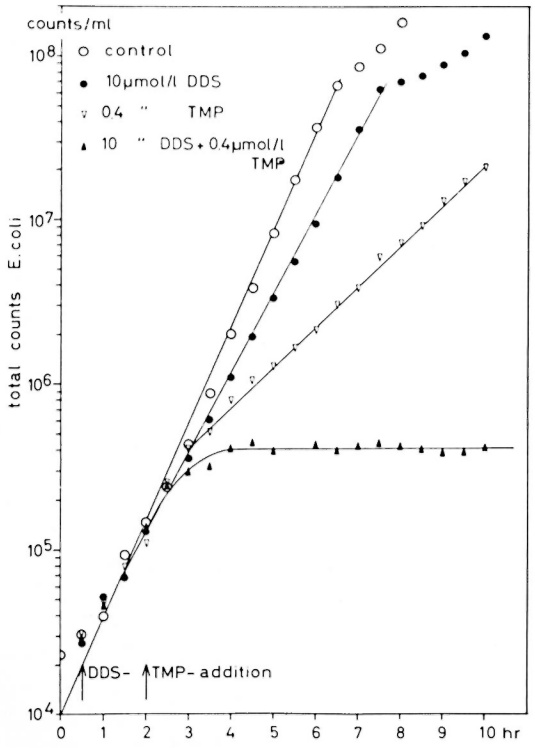


FIG. 13. Typical generation rate curves of *E. coli* (mutaflor) at 37°C in the presence of DDS and TMP alone and in combination. The curves, final drug concentrations (µmol/l) and generation rate constants $k_{app} \times 10^{-4}$ [sec^{-1}] were as follows: (x) control 3.766; (●) 10 DDS, 3.096; (○) 0.4 TMP, 3.25; (△) 10 DDS, 0.4 TMP ± 0.0.

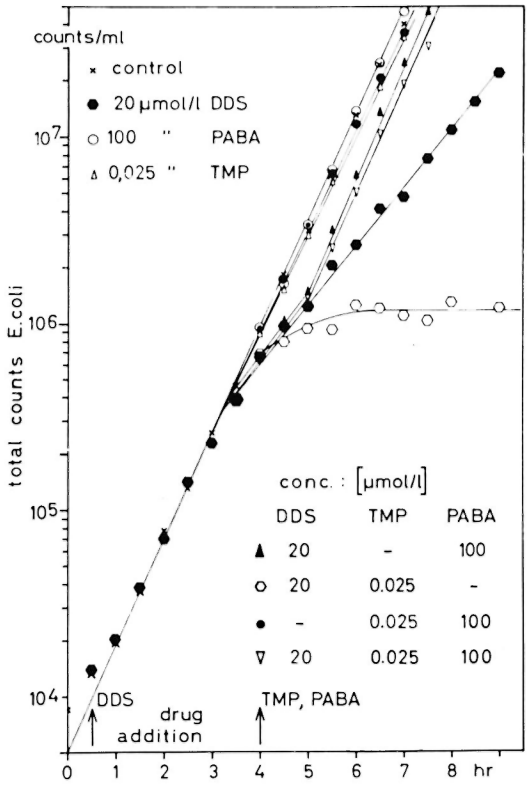


FIG. 14. Typical generation rate curves of *E. coli* (mutaflor) at 37°C in the presence of DDS, PABA and TMP alone and in combination.

dihydropteroic acid synthetase. It is, however, interesting to note that DDS shows a higher affinity towards *E. coli* dihydrofolate reductase than SA whereas the affinity for the main target enzyme dihydropteroic acid synthetase, is opposite as shown above. One could speculate, therefore, that in the case of *M. leprae* the affinity of DDS towards dihydrofolate reductase might even be larger, thus causing an inhibiting effect in addition to the inhibition of dihydropteroic acid synthetase. The two phases of inhibition of DDS seen in *E. coli* cultures could also indicate that there is an inhibitory effect of the analog formed by DDS and phosphorylated dihydropteridine alcohol (see reaction scheme). An experiment was designed in which PABA was added after 4 hr to a culture inhibited by DDS. After this time a certain amount of the DDS analog has been formed; the inhibitory action of this analog should at least not immediately be antagonized by the addition of PABA, which has no affinity for the dihydrofolate reductase, and could show a synergistic or additive effect in combination with TMP. The results are given in Fig. 14 together with the curves obtained in the presence of DDS alone and in a mixture with TMP. It is clearly demonstrated that at least in the case of *E. coli*, the DDS inhibitory activity can be totally antagonized by PABA, i.e., there seems to be no additional activity of the DDS analog or DDS alone at the dihydrofolate reductase level.

Hypothesis about an additional mechanism of action of DDS in *M. leprae*. It seems reasonable to assume that DDS acts on the folate synthesizing pathway of *M. leprae* in a similar way as shown for *E. coli*, especially if one considers the results obtained for other bacterial strains and even for plasmodia. The reported extremely high inhib-

TABLE 3.

Inhibition constants of sulfonamide for E.coli dihydrofolate reductase and E.coli dihydropteroate synthetase

Compound	K_1 (M) Dihydropteroate synthetase	K_1 (M) Dihydrofolate reductase	K_1^2
Sulfaquinoxaline		1.3×10^{-4}	$1.7 \pm 0.4 \times 10^{-4}$
4,4'-Diaminodiphenyl-sulfone		1.8×10^{-4}	$3.0 \pm 1.1 \times 10^{-4}$
Sulfamerazine	4.0×10^{-6}	4.0×10^{-4}	$6.3 \pm 2.2 \times 10^{-4}$
Sulfapyridine	1.3×10^{-5}	5.7×10^{-4}	$1.7 \pm 1.2 \times 10^{-3}$
Sulfathiazole	2.3×10^{-6}	7.4×10^{-4}	$9.6 \pm 4.8 \times 10^{-4}$
Sulfamethoxazole		2.0×10^{-3}	$2.3 \pm 0.3 \times 10^{-3}$
Sulfinilic acid	8.3×10^{-6}	3.5×10^{-3}	$6.5 \pm 0.8 \times 10^{-3}$
Sulfaguanidine	1.7×10^{-5}	3.9×10^{-3}	5.6×10^{-1}
Sulfisoxazole		6.5×10^{-3}	$3.0 \pm 0.3 \times 10^{-3}$
Sulfanilamide	6.7×10^{-5}	2.4×10^{-2}	$1.3 \pm 0.7 \times 10^{-2}$

From: M.Poe: Science 194, 533 (1976)

itory power of DDS against *M. leprae* might be explainable by assuming one or a combination of the following possibilities:

1) a very high affinity to the enzyme dihydropteroic acid synthetase. This does not seem to be very likely from the experience with reversible competitive inhibitors. In all cases known so far, the affinity constant of a strong inhibitor was in the order of magnitude of the natural metabolite.
2) a very low concentration of the competing PABA within the bacterial cell. Since the MED values of DDS against *M. leprae* are extraordinarily low, this is not very likely.
3) an additional inhibitory effect of DDS or the formed analog at the dihydrofolate reductase site or 4) a synergistic effect of DDS or the formed analog at the dihydrofolate reductase. These arguments are still likely to have some relevance even if no evidence can be derived from the studies on *E. coli* as discussed above. Studies on *M. lufu* with a high sensitivity for DDS are in progress in our laboratory to further address these points.
5) accumulation of DDS within bacterial cells of *M. leprae* (deep compartment). In experiments with *M. kansasii*, Panitch and Levy found a 14–15-fold accumulation of DDS within the bacterial cells after 8 days of treatment of the bacterial culture ([17]).
6) an additional action outside of the folate synthesizing enzyme system.
7) The extremely slow generation rate of *M. leprae* and folate metabolism is the rate determining step. Inhibition of folate metabolism by DDS might be very powerful under these circumstances.

SUMMARY

The antibacterial activity of DDS has been studied in whole cell (*E. coli*), cell-free folate synthesizing enzyme extracts and compared to effects obtained for sulfonamides (SA). It is shown that DDS acts as a synthetase inhibitor in the folate syn-

thesizing enzyme system. DDS reacts with the substrate 7,8-dihydro-6-hydroxymethylpterinopyrophosphate to form a 7,8-dihydropteroic acid analog. Bacterial growth kinetic studies were performed to test for possible synergistic activity of the analog in combination with DDS. Possible reasons for the extremely large inhibitory power of DDS against *M. leprae* are discussed.

RESÚMEN

Se estudió la actividad antibacteriana del DDS en células totales (*E. coli*) y en extractos libres de células que contenían las enzimas necesarias para la síntesis del folato. Los resultados se compararon con los obtenidos con sulfonamidas (SA). Se encontró que el DDS actúa como inhibidor en el sistema enzimático que participa en la síntesis del folato. El DDS reacciona con el substrato 7,8-dihidro-6-hidroximetil-pterin-pirofosfato para formar un análogo del ácido 7,8-dihidropteroico. También se hicieron estudios sobre la cinética del crecimiento bacteriano para probar la posible actividad sinergística del análogo en combinación con el DDS. Se discuten algunas posibles razones para explicar el gran poder inhibitorio del DDS sobre el *M. leprae*.

RÉSUMÉ

L'activité anti-bactérienne de la DDS a été étudiée sur des cellules entières de *E. coli*, ainsi que sur des extraits enzymatiques dépourvus de cellules et synthétisant le folate. Cette activité a été comparée aux effets obtenus avec les sulfonamides. On a ainsi montré que la DDS agit comme inhibiteur de la synthétase, dans le système enzymatique synthétisant le folate. La DDS réagit avec le 7,8-dihydro-6-hydroxymethylpterinpyrophosphate, pour former un analogue de l'acide 7,8-dihydropteroïque. Des études cinétiques de la croissance bactérienne ont été menées afin d'étudier l'activité synergique éventuelle de cet analogue en combinaison avec la DDS. Les raisons qui pourraient expliquer le pouvoir inhibiteur extrêmement puisant de la DDS contre *M. leprae*, sont discutées.

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