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Sulfonamide Structure–Activity Relationships in a Cell-Free System. 2. Proof for the Formation of a Sulfonamide-Containing Folate Analog†

Lothar Bock, George H. Miller,‡ Klaus-J. Schaper, and Joachim K. Seydel*

Department of Pharmaceutical Chemistry, Borstel Research Institute, 2061 Borstel, West Germany. *Received May 18, 1973*

Experiments were performed with partially purified "folate" synthesizing enzymes, which support the assumption that sulfonamides are incorporated into folate analogs. An analog, N^1-3-(5-methylisoxazolyl)-N^4-(7,8-dihydro-6-pterinylmethyl)sulfanilamide (VI), has been synthesized using sulfamethoxazole as a model sulfonamide. The identity of the chemically synthesized compound with a ^{35}S-labeled product isolated from cell-free reaction mixtures is demonstrated. In addition, the same "folate" analog could be isolated and identified in growing cultures of *Escherichia coli* after incubation with ^{35}S-sulfamethoxazole.

Indirect evidence that sulfonamides (SA) are incorporated into folate-like compounds by cell-free bacterial extracts has been reported previously by others.[1,2] These conclusions have been derived from experiments in which sulfathiazole was preincubated with pteridine substances and the extract prior to the addition of *p*-aminobenzoic acid (PABA). When the concentration of pteridine is relatively low, a degree of inhibition is found which is much greater than that found when all substrates and inhibitors are combined simultaneously. If, however, a high concentration of pteridine is used, preincubation results in a much smaller degree of inhibition. These experiments have been explained by assuming that the sulfonamide reacts enzymatically with the pteridine substrate during the preincubation. Therefore, when PABA is subsequently added, there is, at low pteridine concentrations, very little remaining pteridine or at high pteridine concentrations there is very little remaining inhibitor. We have repeated these experiments with sulfathiazole and sulfamethoxazole and have obtained similar results. In radioactive tracer experiments with partially purified enzymes we were able to isolate the folate analog by thin-layer chromatography. The chromatographic identity of the analog with a synthetic analog was established.

Experimental Section

General. Melting points were taken with a Leitz melting point microscope and are uncorrected. The nuclear magnetic resonance spectra were determined using a Varian HA-100 spectrometer with tetramethylsilane as internal standard. A Zeiss PMQ II and a Cary 14 spectrometer were used to obtain the ultraviolet spectra. For the extract and enzyme preparation the following equipment was used: MSE-ultracentrifuge Superspeed 65; LKB fraction collector (Ultrorac 7000), equipped with Uvicord II (280 nm) placed in a Colora refrigerating box; Serva column (25 × 900 mm) equipped with a cooling jacket and filled with Sephadex G-100 (25 × 870 mm); and a peristaltic pump LKB Perpex, silicon rubber tube i.d. 1.10 mm, giving a drop rate similar to a hydrostatic pressure of 500 mm. An MSE ultrasonic disintegrator (500 W) was used for the ultrasonication of the bacterial cell mass. Protein solutions were concentrated using an Amicon ultrafiltration system (UF-52; UM-10). The thin-layer radiograms were scanned with a Berthold Scanner equipped with a Siemens methane-flow counter.

Separation of the Folate Synthesizing Enzyme Activities. Lyophilized cell-free extracts (fraction I) were prepared by the method reported previously[3] with the following modifications. Cells (*E. coli* mutaflor) were broken by ultrasonication in ten intervals of 20 sec at a temperature of 5°. The treatment with ribonuclease was omitted. Fraction I was further purified and separated on Sephadex G-100 into two enzymatic activities (Figure 1, 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase, E_1, and 7,8-dihydropteroate synthetase, E_2). Purification was accomplished by dissolving 170–200 mg of the lyophilisate in 3 ml of 0.01 *M* Tris-HCl buffer, pH 8. Insoluble particles were removed by centrifugation and the resulting supernatant was chromatographed at 4° on a Sephadex G-100 column. Fractions of 50 drops were separated (4 drops per min). Molecular weights of approximately 20,000 for the E_1 activity (kinase) and 45,000 for the E_2 activity (synthetase) were obtained by this fractionation procedure. Richey and Brown[4] have reported 15,000 and 50,000, respectively. Fractions which contained maximum E_1 and E_2 activity (fractions 86–97 and 63–74, respectively) were collected from three chromatographic runs, filtered through a 0.2-μ membrane filter (Sartorius), concentrated by ultrafiltration, and lyophilized. In the experiments with ^{35}S-sulfamethoxazole the protein concentrations of the solutions with E_1 activity and with E_2 activity which were employed were 4.5 and 1.9 mg/ml, respectively. Protein concentration was determined by the method of Folin and Lowry,[5] using bovine serum albumin as a standard.

Test Method for the Determination and Localization of Kinase (E_1) and Synthetase Activity (E_2) after Chromatographic Separation of the Folate Synthesizing Extract on Sephadex G-100. Folate growth equivalents as described previously[3] were used to assay enzymatic activity. The enzymatic activity of E_2 fractions (50–75) was determined by combining them with a corresponding volume of the pooled E_1 fractions. The standard reaction mixture of 450 μl contained 90 m*M* Tris-HCl (pH 8), 20 m*M* $MgCl_2$, 45 m*M* 2-mercaptoethanol, 1.0 m*M* ATP, 18 m*M* PABA, 36 m*M* 7,8-dihydro-6-hydroxymethylpterin, 15 μl of pooled fractions of E_1 activity, and 5 μl of the fractions of E_2 activity. Before use fractions of E_1 and E_2 activity were sterilized by filtration through 0.2-μ membrane filters. The reaction mixtures were prepared aseptically and incubated at 37° for 4 hr. Synthesis was stopped by addition of 5 μl of 50% trichloroacetic acid (TCA) and neutralized by 20 μl of 1 *N* NaOH. To determine the amount of folate-like product, three aliquots (20, 50, and 100 μl) were taken from these reaction mixtures and the amount of "folate" was determined microbiologically with *Streptococcus faecalis* ATCC 8043.[3] A typical example is given in Figure 1. The exact location of E_1 activity was performed in a similar manner except that for this experiment the pool of the previously located E_2 activity (15 μl) was combined with fractions of the expected E_1 activity (5 μl).

Thin-Layer Chromatography. Both thin-layer chromatography and radiochromatography were performed on 0.25-mm cellulose plates (Macherey and Nagel, Germany) or on plates prepared with a 0.4-mm layer of Silica Gel F_{254} (E. Merck, Germany). The solutions or reaction mixtures (10 or 50 μl) were spotted. Chromatograms were developed for 12 cm using either aqueous solutions of Na_2HPO_4 (5%, w/v), LiCl (0.21%, w/v), NH_4Cl (3%, w/v) or methyl ethyl ketone + pyridine, 75 + 5 (volume), as solvent system. After the plates were developed, the spots were viewed with either short wave ultraviolet (254 nm) or, in the case of the ^{35}S-sulfamethoxazole containing reaction mixtures, with a thin-layer scanner (Berthold) equipped with a Siemens methane-flow counter, diaphragm slit 45 × 2 mm.

Identification of a Sulfonamide-Containing Folate Analog in Whole Cell Bacteria. ^{35}S-Sulfamethoxazole was added to growing cultures and after 12 hr of incubation the cells were harvested by filtration (0.2-μ membrane filter). The cells were washed on the filter with isotonic NaCl solution and broken using a simple glass homogenizer and glass powder. The resulting paste was diluted wih a small amount of phosphate buffer (pH 7.4, 0.01 *M*) and centrifuged at 15,000 rpm for 6 min (Eppendorf centrifuge 3200). The supernatant was spotted immediately on cellulose plates and developed with Na_2HPO_4 solution (5%).

Determination of Sulfonamide Inhibitory Activities in the Cell-Free System. The ratio of the amount of folate produced in reaction mixtures (reactants and concentrations as described

†Dedicated to Alfred Burger, a prominent contributor to medicinal chemistry.

‡Medical College of Virginia, School of Pharmacy, Richmond, Va. 23298.

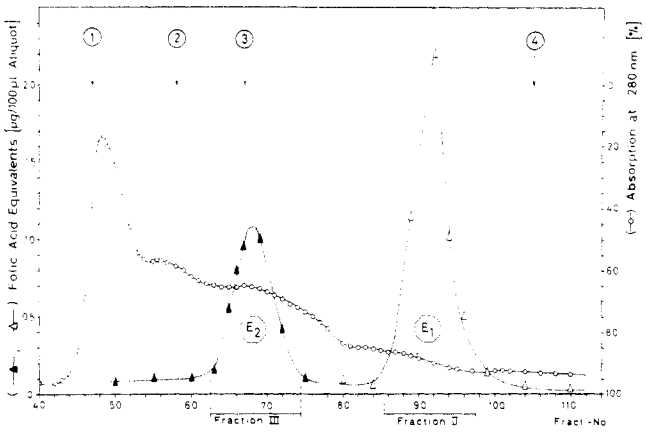


Figure 1. Elution profile of the pteroate synthesizing enzyme system from *E. coli*. Chromatography on Sephadex G-100. The protein absorption was determined with LKB-Uvicord II. The enzymatic activity of the single fractions in the range of fractions 50–75 and 80–110 was determined as described in the Experimental Section. E_1 = 7,8-dihydro-6-hydroxymethylpterin pyrophosphokinase activity; E_2 = 7,8-dihydropteroate synthetase activity. Substances for calibration: (1) blue dextrane (Pharmacia, Uppsala, Sweden), (2) bovine serum albumin, (3) egg albumin, (4) cytochrome c (2–4: Boehringer, Mannheim, Germany).

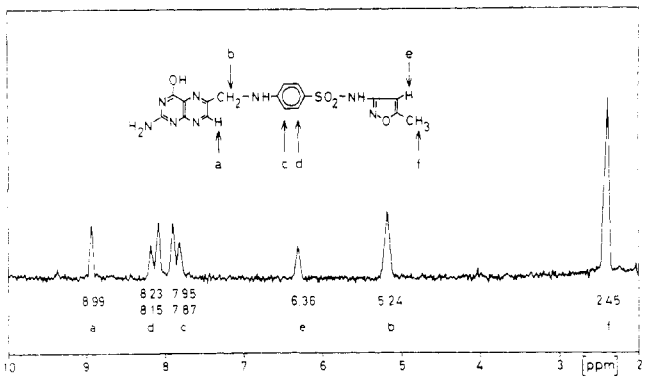


Figure 2. Nmr spectrum of N^1-3-(5-methylisoxazolyl)-N^4-(6-pterinylmethyl)sulfanilamide (V) in CF_3COOD, 100 MHz; tetramethylsilane used as internal standard.

above) containing sulfonamide to that produced in controls was used as fractional activity (a). The concentration of sulfonamide causing a 50% inhibition of folate synthesis was obtained by plotting $i = 1 - a$ *vs.* the logarithm of sulfonamide concentration. 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 sulfonamide causing a 50% inhibition in rate of folate synthesis was evaluated from plots of $1/(k_0 - k_i)$ *vs.* the reciprocal of the sulfonamide concentration where k_0 and k_i are the rate constants for folate synthesis in the absence and presence of inhibitor. A detailed experimental description was previously published.[3]

Assay of Folate Growth Equivalents. The methods used have already been described.[3]

Sulfonamides. Sulfamethoxazole was the gift of the Burroughs Wellcome Co. ^{35}S-Sulfamethoxazole was a generous gift of the Deutsche Hoffmann-La Roche. N^1-Phenylsulfonamides were prepared by standard methods which have been described elsewhere.[6]

Pteridine Derivatives. N^1-3-(5-Methylisoxazolyl)-N^4-(6-pterinylmethyl)sulfanilamide (V). (a) Triose reductone[7] (I, 0.4 g, 4.5 mM) was dissolved in 25 ml of H_2O and a solution of sulfamethoxazole (II, 1.1 g, 4.3 mM) in 250 ml of 0.5 N HCl was added dropwise. The mixture was stirred at 20° for 12 hr. Filtration afforded 0.85 g (III) as a yellow precipitate with mp 210–230°, yield 60%.

(b) The crude Schiff base (III, 0.85 g, 1.85 mmol) was refluxed with 6-hydroxy-2,4,5-triaminopyrimidine sulfate hydrate (IV, EGA, Germany) (0.5 g, 1.95 mmol) and anhydrous, pulverized NaOAc in 20 ml of EtOH for 4 hr. The mixture was suspended in water, stirred for 5 min, and filtered. The residue was dissolved in a small amount of 0.1 N NaOH, placed (in 5–10-ml aliquots) onto a Sephadex G-10 column (25 × 530 mm), and eluted with 0.1 N NaOH. After a preliminary fraction of 60 ml the extinction of the eluate fractions (3 ml) was followed at 260 and 365 nm. A maximum was observed for fractions 100–110. Fractions 70–145 were collected and, after adjustment of the pH to 4–5, kept in refrigerator for 12 hr. The precipitate was collected, washed with H_2O and EtOH, and dried at 60°: yield 250 mg of V (31%); mp >350°; uv (0.1 N NaOH) 262 nm (ϵ 28,400), 370 (10,100); nmr (CF_3COOD, 100 MHz) δ 8.99 (s, 1 H), AA′BB′ centered at 8.19 and 7.91 (J = 8 Hz, 4 H), 6.35 (s, 1 H), 5.24 (s, 2 H), 2.45 (s, 3 H) (Figure 2).

The 6 position of the pteridine ring substituent was proven by oxidation of V with $KMnO_4$ according to Roth, *et al.*[8] The uv spectrum of the oxidation product was identical with that of pterin-6-carbonic acid.[9]

N^1-3-(5-Methylisoxazolyl)-N^4-(7,8-dihydro-6-pterinylmethyl)sulfanilamide (VI). Reduction of V was performed with $Na_2S_2O_4$ as described by Friedkin, *et al.*,[10] with minor modifications: uv (0.1 N NaOH) 235 nm (ϵ 16,300), 272 (18,500), 320 (6100).

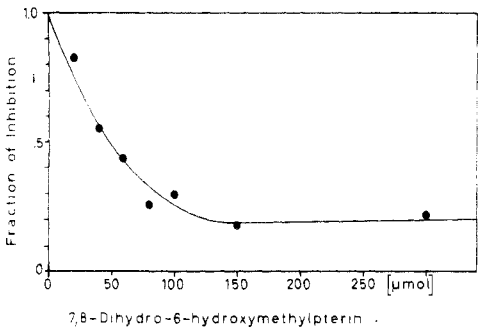


Figure 3. Fraction of inhibition, i, of folate synthesis at various concentrations of 7,8-dihydro-6-hydroxymethylpterin and constant sulfamethoxazole concentration. The sulfonamide (5 μM) was preincubated with various concentrations of pterin substrate and extract for 3 hr before allowing folate synthesis. Folate synthesis was started by adding PABA; synthesis was stopped after 3 hr. All other conditions were as described in the Experimental Section.

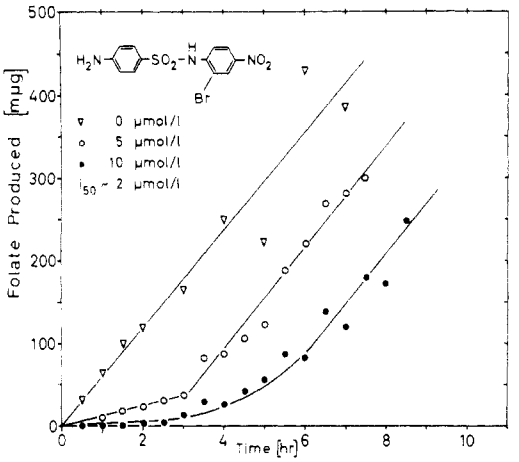


Figure 4. Kinetics of inhibition of folate synthesis by N^1-2-bromo-4-nitrophenylsulfonamide. The highly active N^1-2-bromo-4-nitrophenylsulfonamide only exhibited pseudo-zero-order kinetics for a short period of time. After this the rate of folate synthesis is similar to that seen in the control.

Results and Discussion

Figure 3 gives the results of a typical attempt to show the phenomenon of analog formation. The degree of inhibition caused by a fixed concentration of the sulfonamide is plotted *vs.* the concentration of dihydropterin alcohol which was preincubated for 3 hr with the sulfonamide and the extract. The decreasing inhibition observed with increasing dihydropterin alcohol concentration can be explained by postulating extract catalyzed folate analog for-

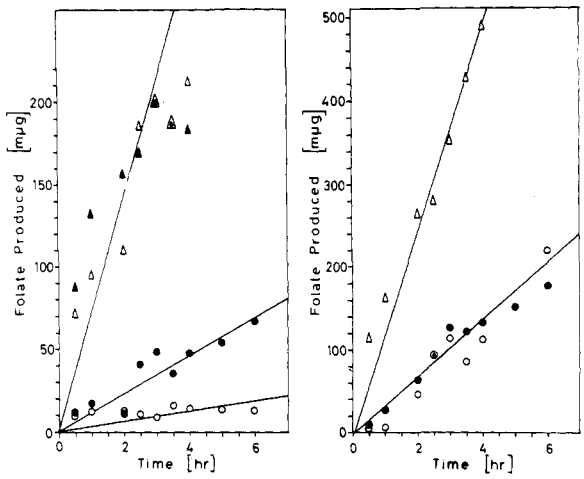


Figure 5. Kinetics of inhibition of folate synthesis by N^1-4-nitrophenylsulfonamide with and without preincubation. (a, left) Kinetics of inhibition where the sulfonamide (12 μM) and 7,8-dihydro-6-hydroxymethylpterin (32 μM) were preincubated for 3 hr and PABA (20 μM) was subsequently added (○○○) and where sulfonamide (12 μM), pterin component (32 μM), and PABA (20 μM) were added together to the reaction mixture at zero time (●●●). The appropriate controls where no sulfonamide was added are △△△ and ▲▲▲. (b, right) Kinetics of inhibition by N^1-4-nitrophenylsulfonamide as under (a); however, the concentration of 7,8-dihydro-6-hydroxymethylpterin was 54 μM (○○○ and ●●●, control △△△).

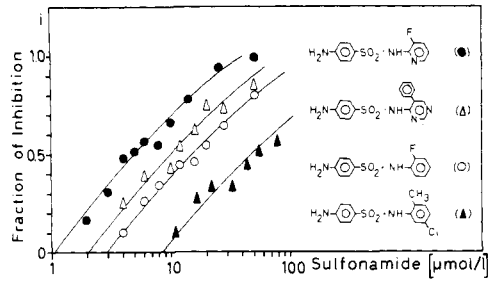


Figure 6. Determination of the inhibitory activity of several N^1-substituted sulfonamides by the single point method. Plots of the fraction of inhibition, *i*, of cell-free folate synthesis *vs.* log of the sulfonamide concentration. The conditions of the experiments were described in the Experimental Section.

mation during the preincubation as was done in similar experiments with sulfathiazole.[1,2] Similar indirect evidence for the formation of a sulfonamide-containing folate analog in the cell-free experiments is provided by the experiments in which the kinetics of inhibition were studied for a relatively long time as described previously.[3] The observation that the rate of folate synthesis returns to a rate of synthesis equal to that of the control, after an initial period of inhibition by this highly active sulfonamide (N^1-2-bromo-4-nitrophenylsulfonamide), is consistent with the idea that such reaction mixtures no longer contain any inhibitor (Figure 4). Of course, removal or destruction of the inhibitor by any other mechanism would lead to a similar type of kinetics. However, the inhibitors employed are stable under the pH and temperature conditions used and in the preincubation experiments the presence of both the extract and the pteridine is required.

Figure 5 shows the results of a kinetic experiment with N^1-4-nitrophenylsulfonamide where the sulfonamide was preincubated with pteridine substrate and the extract prior to the addition of PABA. A degree of inhibition is found which is much greater than that found when all substrates and the inhibitor are combined simultaneously (Figure 5a). If the amount of the pteridine substrate (6-hydroxymethyldihydropterin) is 1.7 times greater (Figure 5b), the degree of inhibition in the preincubation experiment is identical with the experiment where all substrates and the inhibitor are added simultaneously. Additional indirect evidence that sulfonamides are functioning in this system by reacting enzymatically with the 7,8-dihydro-6-hydroxymethylpterin is provided by the nature of the dose–response curves obtained.[3] The usual dose–response curve obtained in cases of competitive inhibition has a rather large concentration range. That is, the ratio of the dose causing a 10% inhibition to that causing a 90% inhibition is often in the order of 1:100. The obtained dose–response curves for several sulfonamides (Figure 6) have a small range of inhibitory concentrations. The ratio of doses causing a 10% to that causing a 90% inhibition falls between 1:5 and 1:20 for the compounds studied. Such a narrow range may often be associated with inhibition caused by depletion of a substrate.[11] This, indeed, is to be expected if the sulfonamides are reacting with the pteridine substrate in addition to competing with PABA for binding sites.

Table I. Dependency of Inhibition of "Folate" Synthesis by Sulfamethoxazole on PABA Concentration When PABA is in Excess[a]

Concn of PABA, μM	Obsd I_{50}, μM	I_{50}/[PABA]
20	15	0.75
50	45	0.90
100	85	0.85

[a] Experimental conditions were as described in the Experimental Section except that the extract used for this experiment had not been chromatographed on Sephadex G-25.

Table II. Dependency of Inhibition of "Folate" by Sulfamethoxazole on PABA Concentration When PABA is Present in a Limited Concentration[a]

Concn of PABA, μM	Concn of sulfamethoxazole, μM	Folate produced in control, ng	Folate produced in reaction, ng	Obsd I value
0.00[b]	10	18	9.9	0.45
1.25	10	35	28.0	0.20
2.50	10	57	43.0	0.24
3.75	10	141	89.0	0.37
5.00	10	162	99.0	0.45
7.50	10	205	141.0	0.31
10.00	10	278	214.0	0.27

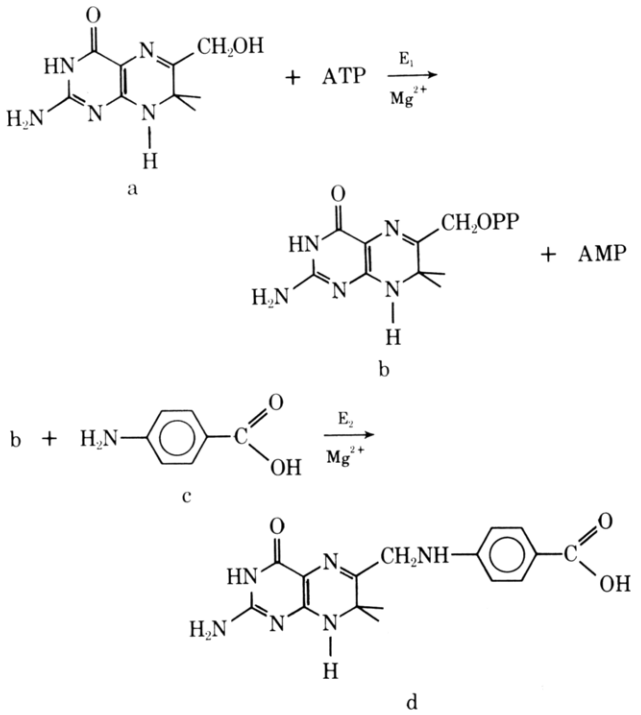
[a] Experimental conditions as described in the Experimental Section except that the concentration of PABA was varied as indicated. [b] A small quantity of endogenous PABA is obviously present in the extract even after chromatography on Sephadex G-25.

At high PABA concentrations the degree of inhibition observed for a given sulfonamide concentration is a function of the PABA concentration. Table I gives the results of an experiment in which the I_{50} of sulfamethoxazole was determined at several high PABA concentrations. A relatively constant I_{50} to PABA concentration ratio is observed. Brown[1] has reported data of this type for several other sulfonamides. However, at lower PABA concentrations the degree of inhibition observed at a given sulfonamide concentration tends not to be decreased by small increases in the PABA concentration as seen in Table II. These experimental data can be explained if one assumes that during the initial time period the enzyme is occupied principally by the large excess of sulfonamide. After the sulfonamide has been incorporated into a folate-like product the enzyme is free to synthesize for a limited and con-

stant time (under the experimental conditions) the normal folate product. Therefore, the degree of inhibition would be a function of the time required to deplete the sulfonamide and no longer completely dependent on the ratio of substrate to inhibitor.

Identification of a Sulfonamide-Containing Folate Analog in a Cell-Free System. Brown[1] has reported more direct evidence showing that sulfanilic acid is incorporated by an *E. coli* extract into a new compound in the presence of 7,8-dihydro-6-hydroxymethylpterin and ATP-Mg^{2+}. He showed chromatographically that ^{35}S-sulfanilic acid is changed into two compounds with different R_f values under the normal reaction conditions. Because sulfanilic acid is an unusual compound, which inhibits folate synthesis in the cell-free system but does not have any antibacterial activity, and because the radioactive spots were not positively identified, we have repeated these experiments with ^{35}S-sulfamethoxazole in a cell-free "folate" synthesizing extract. The biosynthetic pathways for dihydropteroic acid are assumed to proceed in *E. coli* in accordance with that shown in Scheme I.[12-14] Prior to attempts to isolate the sulfonamide-containing analog from cell-free systems, we synthesized the expected analog according to Scheme II.[7,10,15]

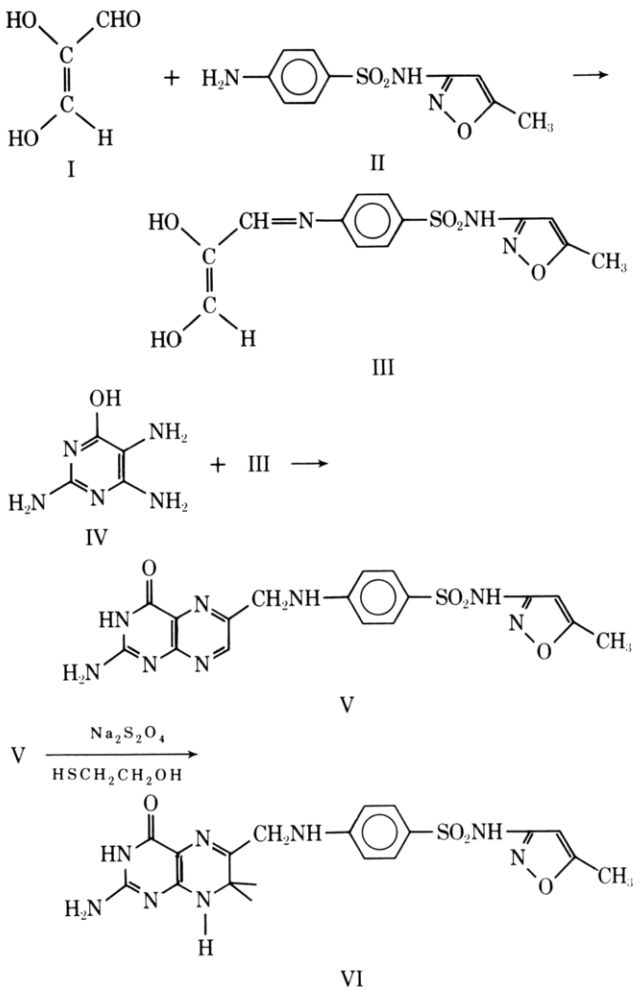
Scheme I



Reductone (I) is condensed with sulfamethoxazole (II) to form the corresponding Schiff base (III), which then reacts with triaminohydroxypyrimidine (IV) to form N^4-(6-pterinylmethyl)sulfamethoxazole (V). The nmr is in agreement with the assigned structure (Figure 2). Substitution in the 6 position of the pteridine ring was proven by oxidation of compound V with $KMnO_4$[8] in NaOH which results in the formation of pterin-6-carbonic acid, identified by uv-spectral analysis.[9] The reduction to N^4-(7,8-dihydro-6-pterinylmethyl)sulfamethoxazole (VI) was performed by treatment with $Na_2S_2O_4$ in the presence of 2-mercaptoethanol.

Since it was assumed that the sulfonamide-containing folate analog would be synthesized in the enzyme mixture in small amounts, thin-layer chromatography (tlc) was utilized for identification. As this product is insoluble in most organic solvents, aqueous systems and cellulose

Scheme II



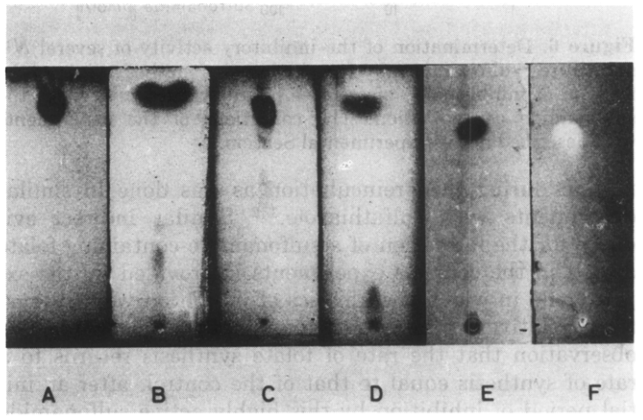


Figure 7. Thin-layer chromatogram of sulfamethoxazole (SA) and the ^{35}S-containing pterin analogs V and VI. Typical thin-layer chromatograms of sulfamethoxazole (II, A and F) and of mixtures of compounds II, V, and VI on cellulose layer (CEL 300, UV_{254}, 0.25 mm, Macherey and Nagel, Germany), developed by Na_2HPO_4 (5%, A and B), LiCl (0.21%, C), NH_4Cl (3%, D), and on silica gel (F_{254}, 0.25 mm, Merck AG, Germany) developed by methyl ethyl ketone and pyridine (75 and 5 v/v, E and F). R_f values are: (A and B) II, 0.87, V, 0.385, VI, 0.262; (C) II, 0.85, V, 0.715, VI, 0.54; (D) II, 0.85, V, 0.1, VI, 0.1; (E and F) II, 0.58, V, 0.00, VI, 0.00.

plates gave the best results. However, silica gel plates were used when the solvent was methyl ethyl ketone. Figure 7 shows typical thin-layer chromatograms for the synthetic folate analog in four different solvent systems which cover a wide range of R_f values. Figure 7 also gives the R_f

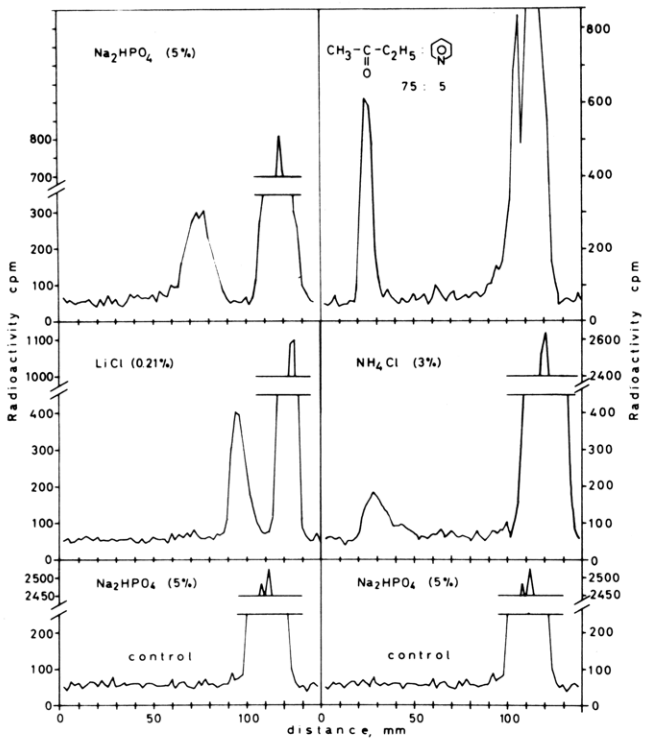


Figure 8. Thin-layer radiochromatogram of cell-free folate synthesizing reaction mixtures containing ^{35}S-sulfamethoxazole. The solvent systems used were: Na_2HPO_4 (5%), LiCl (0.21%), NH_4Cl (3%) on cellulose layers, and methyl ethyl ketone and pyridine (75 and 5 v/v) for silica gel. The radiochromatograms demonstrate the separation of an additional radioactive spot. The R_f values are identical with the R_f values found for N^1-3-(5-methylisoxazolyl)-N^4-(7,8-dihydro-6-pterinylmethyl)sulfanilamide (VI) under the same conditions (Figures 7 and 9). The control reaction mixture, which did not contain the pterin substrate, shows the normal position of SA in the chromatographic system.

values for sulfamethoxazole in the same solvent systems. There is nearly no change in sulfamethoxazole R_f values. Therefore, using these systems we could separate the expected sulfamethoxazole folate analog from unchanged sulfamethoxazole and other components of the incubation mixture.

The influence of extract proteins on the R_f values was studied before starting the radiochromatography of cell-free reaction mixtures incubated with ^{35}S-sulfamethoxazole. The amount of extract employed in these systems was found to have a large influence on the R_f values of the sulfonamide analog. This apparently resulted from an incomplete separation of the proteins after the reaction mixtures were stopped with TCA. In addition, radioactive material was lost with the precipitated protein. For this reason, the extract proteins were further purified as described in the Experimental Section. Two enzyme fractions which show high "folate" synthesizing activity on combination have been obtained (Figure 1).

Thin-Layer Radiochromatograms. Typical radiochromatograms of reaction mixtures combined with ^{35}S-sulfamethoxazole are given in Figure 8. A new radioactive compound is evident in each of the four solvent systems which is not present in the control reaction. Control reactions were prepared identically except for the omission of 7,8-dihydro-6-hydroxymethylpterin. The R_f values of the radioactive product in the reaction mixtures and those of the synthesized analog were identical in the four different solvent systems used for chromatography (Figure 7). Because a slight change in the R_f value of the pterin derivative in the presence of extract proteins and ATP was still observed, the synthesized analog VI was added to the

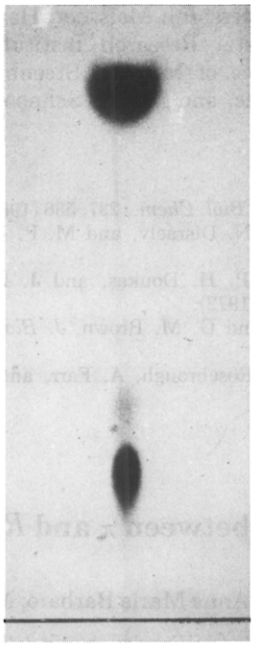


Figure 9. Autoradiography of a reaction mixture containing ^{35}S-sulfamethoxazole. Autoradiography of a reaction mixture containing ^{35}S-sulfamethoxazole after chromatography on cellulose layer (Macherey and Nagel, Germany), developed by Na_2HPO_4 (5%, w/v); 10 μl of the reaction mixture was spotted. Incubation time was 5 hr. Compound V (R_f 0.39) and compound VI (R_f 0.25) are clearly separated (type of film: Kodak safety film RPR□, exposure time 48 hr).

reaction mixtures containing ^{35}S-sulfamethoxazole and the spots were viewed under ultraviolet light. The R_f values of these spots were identical with the maximum of ^{35}S radioactivity. If a smaller volume was spotted on the thin-layer plates, a better separation was obtained and an additional radioactive spot could be detected, which was only incompletely separated in the other chromatograms. Figure 9, for example, shows a contact autoradiography of a chromatogram where 10 μl of the reaction mixture was spotted on a cellulose plate and developed with a Na_2HPO_4 solution. The additional radioactive spot is identical in R_f value with N^1-3-(5-methylisoxazolyl)-N^4-(6-pterinylmethyl)sulfanilamide (V). If the reaction mixtures are not protected by N_2 during the reaction this spot becomes enlarged. It is highly probable that the additional radioactive spot reported in the paper of Brown[1] in the study with ^{35}S-sulfanilic acid is also the oxidized form of the pterinyl analog.

Identification of a Sulfonamide-Containing Folate Analog in Whole Cell Bacteria. In addition to the results with ^{35}S-sulfamethoxazole in cell-free systems which clearly demonstrate the biosynthesis of the pterinyl analog VI, similar experiments were performed with growing cultures of *E. coli* where a permanent de novo synthesis of 7,8-dihydro-6-hydroxymethylpterin pyrophosphate takes place. ^{35}S-Sulfamethoxazole was added to the growing cultures. After 12 hr of incubation the cells were harvested and a cell-free extract was prepared as described in the Experimental Section. Tlc of this extract gave spots on the contact autoradiogram which were identical in R_f value with those obtained from cell-free systems (^{35}S-sulfamethoxazole, 0.91; V, 0.37; VI, 0.24). These experiments demonstrate that sulfonamides compete with PABA not only for binding sites but also for the pterin substrate in both cell-free systems and in growing cultures of *E. coli*.

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Relationship between π and R_m Values of Sulfonamides†

Gian Luigi Biagi,* Anna Maria Barbaro, Maria Clelia Guerra, Giorgio Cantelli Forti, and Maria Enrica Fracasso

Cattedra di Farmacologia e Farmacognosia dell'Università di Bologna, Bologna, Italy. *Received July 6, 1973*

The chromatographic R_m values of sulfonamides in several reversed phase tlc systems were shown to be very well correlated with the Hansch π values in an isobutyl alcohol-water system. On the other hand, when the π values were calculated from the partition data obtained with chloroform, toluene, or ethylene dichloride as organic phases the correlation coefficients were much lower. By comparing R_m values of sulfonamides, cephalosporins, and penicillins, it was pointed out that sulfonamides are more hydrophilic than both series of antibiotics. Therefore, sulfonamides and penicillins could be considered as being represented respectively by molecules falling on the left and right side of the theoretical parabola relating lipophilic character and antibacterial activity.

The relation between physicochemical properties and biological activity of sulfonamides has been studied by Bell and Roblin,[1] Cowles,[2] Seydel,[3] Seydel, *et al.*,[4] and many others.[5] However, Fujita and Hansch,[6] Fujita,[7] and Seydel[3a] seemed to obtain the best results. The correlation between biological activity and chemical structure was analyzed by means of substituent constants such as the Hammett σ constant, the pK_a value, and the hydrophobicity constant π. Hansch, *et al.*,[8] defined $\pi = \log P_X - \log P_H$ where P_X and P_H are the partition coefficients, determined in the system 1-octanol-water, of the substituted and unsubstituted compounds, respectively. However, because of the practical difficulties of the determination of the partition coefficient, Boyce and Milborrow[9] had proposed the chromatographic R_m value as an expression of the lipophilic character of molecules. The R_m values resulted to be useful in correlating biological activity and lipophilic character of *N*-*n*-alkyltritylamines,[9] bis(dichloroacetamides) and vitamin K analogs,[10] penicillins,[11a,12] cephalosporins,[11a] and testosterone esters.[11b] The contributions of Martin and Synge,[13] Consden, *et al.*,[14] and Brenner, *et al.*,[15] are very important in understanding the relationship between log P and the chromatographic R_m value.

The partition coefficient P of a solute between two immiscible phases can be expressed by

$$P = C_s/C_m \quad (1)$$

where C_s and C_m are the concentration of solute, at equilibrium, in the organic and aqueous phase, respectively. Since $C_s = q/V_s$ and $C_m = p/V_m$, eq 2 is obtained

$$P = (V_m/V_s)\cdot(q/p) \quad (2)$$

where q and p are the fractions of solute in the organic and aqueous phase, and V_s and V_m are the volumes of the organic and aqueous phase, respectively. However $q + p = 1$ and $q = 1 - p$. Therefore, by substituting and taking the logarithms

$$\log P = \log V_m/V_s + \log (1/p - 1) \quad (3)$$

where V_m/V_s can be taken as an arbitrary constant for a given system.

Martin and Synge[13] and Consden, *et al.*,[14] had shown that in a chromatographic system the partition coefficient can be expressed by

$$\log K = \log A_l/A_s + \log (1/R_f - 1) \quad (4)$$

where A_l/A_s, which is the ratio of the volumes of the mobile and stationary phase, has the same meaning of V_m/V_s in eq 3, and R_f is the ratio of the distances traveled by the solute and the front of the mobile phase. Equation 4 indicates that in reversed-phase tlc the R_f value is related to the partition coefficient of the substance between the nonpolar stationary phase and the polar mobile phase.

Since Brenner, *et al.*,[15] had shown that in a chromatographic system $p = R_f$, eq 3 and 4 are clearly related. A proper choice of the volumes V_m and V_s, A_l and A_s, should make possible to obtain the same value for K and P.

Consden, *et al.*,[14] choose such a water content of the paper that the partition coefficient of several amino acids was close to that found by England and Cohn[16] by means of a direct measurement. Bate-Smith and Westall[17] introduced the term

$$R_m = \log (1/R_f - 1) \quad (5)$$

which cannot be considered as an expression of the true partition coefficient as it does not account for log A_l/A_s. However, it was possible to show very good correlations between R_m and π values,[11b,c,18] the latter being derived from the experimental log P values. In particular, since $\Delta R_m = R_{m(X)} - R_{m(H)}$, where $R_{m(X)}$ and $R_{m(H)}$ are the chromatographic R_m values of the substituted and unsub-

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