J. Pharm. Pharmac., 1974, **26**, 228–234 *Received July 5, 1973*

Relation between structure of sulphonamides and inhibition of H_2-pteroate synthesis in *Escherichia coli*

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Inhibitory activities for cell-free H_2-pteroate synthesis in *E. coli* have been estimated for a heterogeneous series of N^1-substituted sulphonamides and for N^1N^1-dialkyl-sulphonamides. The relation between the cell-free activities and the acid dissociation constants of the N^1-substituted sulphonamides showed a parabolic dependency, indicating that the common parabolic relation between antibacterial activity and acidity of sulphonamides is not, as stated by the Breuckner and Cowles theory, a penetration phenomenon. The extent of ionization of the sulphonamides significantly affects the amount of inhibition of H_2-pteroate synthesis. Evidence has been obtained that not only the ionized sulphonamide molecules interfere with the enzyme system, but the unionized molecules also contribute to affinity for the enzyme. The non-acidic N^1N^1-dialkyl-sulphonamides inhibited cell-free H_2-pteroate synthesis. Some of the N^1N^1-dialkyl-sulphonamides showed higher affinity for the enzyme than their mono-substituted parent compounds. The relations between sulphonamide structure and cell-free activity are explainable by the Bell and Roblin theory.

To explain the parabolic relation between bacteriostatic activity of sulphonamides and their respective pKa's, Bell & Roblin (1942) considered that bacteriostatic activity increased with the negative character of the sulphonyl, $-SO_2-$, group. They postulated that, because of the electron-attracting property of the $-SO_2-$ group, the ionized sulphonamide group, $-SO_2-NR-$, which bears a formal charge, would enhance the electron density of the $-SO_2-$ group. Thus, the ionized sulphonamide molecule would be more active than the unionized molecule. If, however, the electron-attracting power of substituent R increases (as reflected in increasing acidity), competition is set up with the $-SO_2-$ group for the electrons of the amide nitrogen. Hence, the electron-attracting power of substituent R should lie in an optimal range for maximal activity so that the pKa of the sulphonamide is about 6–7.

An alternative explanation was proposed by Breuckner (1943) and by Cowles (1942). They postulated that the sulphonamide molecule exclusively in the ionized form is responsible for the bacteriostatic action but only the unionized form can readily penetrate the bacterial-cell, giving a parabolic relation between pKa and activity.

It has been shown (Brown, 1962; Shiota, Disraely & McCann, 1964; Ortiz & Hotchkiss, 1966; Miller, Doukas & Seydel, 1972; Thijssen, 1973a) that sulphonamides competitively inhibit the synthesis of H_2-pteroate,* the precursor of dihydrofolate, in cell-free systems prepared from microorganisms. Brown (1962) showed that the

* Abbreviations used: H_2-pteroate, 7,8-dihydropteroic acid; PABA, *p*-aminobenzoic acid; H_2-pter, 2-amino-4-hydroxy-6-hydroxymethyl-7,8-dihydropteridine.

H_2-pteroate inhibitory activities of several sulphonamides were about proportional to the antibacterial activities of the compounds. Miller & others (1972) showed that, for two homologous series, N^1-phenylsulphonamides and N^1-pyridylsulphonamides, the inhibitory activities of the compounds, measured in a cell-free system, were linearly related to their antibacterial activities. Further, it was shown that the inhibitory activity increased linearly with the increase in acidity of the compounds. From these results, Miller & others concluded that the parabolic relation between pKa and sulphonamide antibacterial activity is best explained by the Breuckner and Cowles theory. However, the sulphonamides used by Miller & others had a limited pKa range. The pKa of the most acidic sulphonamide used, N^1-(2-bromo-4-nitrophenyl)sulphonamide, 5·70, is near the pKa-range (pKa: 6–7) required for maximal antibacterial activity (Bell & Roblin, 1942; Cowles, 1942; Breuckner, 1943).

To obtain more definite information on this aspect, inhibition studies on the H_2-pteroate synthesizing enzyme system of *E. coli* B were undertaken with a heterogeneous series of sulphonamides having pKa-values from 2·92 to 10·88, and with some N^1N^1-dialkyl-sulphonamides.

MATERIALS AND METHODS

Cell-free extracts

Escherchia coli B was cultured on a synthetic medium, containing (g litre^{-1}): KH_2PO_4, 6; $K_2HPO_4 \cdot 3H_2O$, 6; NH_4Cl, 2; $MgSO_4$, 0·05; glucose, 20; and casamino acids (DIFCO), 10. 4 litre amounts were cultured at 37° with aeration. Cells were harvested in the logarithmic phase and washed with saline. Approximately 12 to 18 g of cell-paste was obtained. The cells were suspended in 60 ml tris-HCl buffer (0·05 M, pH 8·0) and disintegrated by sonication for 2 min at 0° using a Branson sonifier B-12. After sedimentation of the cell-debris by centrifugation, the supernatant was dialysed overnight against 5 litres 0·05 M tris-HCl buffer (pH 8·0) at 4°. All subsequent manipulations were made at 4°. From the undialysed material a 30–70% ammonium sulphate fraction was prepared. The precipitate was collected by centrifugation, resuspended in about 60 ml 0·05 M tris HCl buffer (pH 8·0) and dialysed for 24 h (2 changes of 5 litres buffer). Any precipitated protein was removed by centrifugation and the resulting solution was put into vials (in 2 ml amounts) and stored at −20°. No loss of activity occurred for at least 1 month. The protein content was determined according to Lowry, Rosebrough & others (1951).

Determination of the enzyme activity

The formation of H_2-pteroate by enzyme was determined by a simplified radioassay method (Thijssen, 1973a). The reaction mixtures were prepared to contain in a volume of 0·45 ml: 0·5 μmol ATP; 2 μmol $MgCl_2$; 0·5 mg Na-ascorbate; 0·1 mmol tris HCl buffer (pH 8·0); 25 nmol H_2-pter, and enzyme preparation containing 350 μg of protein. After incubation at 37° for 10 min, 50 μl of a [7-^{14}C] *p*-aminobenzoate solution was added and the incubation was continued. The enzyme reaction was stopped by adding 1·5 ml of a concentrated citric acid—phosphate buffer, pH 3·8, whereafter [^{14}C] PABA, which was not incorporated into H_2-pteroate, was extracted by ether. The radioactivity remaining in the water layer is a quantitative measure of the H_2-pteroate formed (Thijssen, 1973a).

Determination of the Inhibition Index of the sulphonamides

The inhibition of H_2-pteroate synthesis by sulphonamide was followed at 6 inhibitor concentrations in duplicate experiments. The Inhibition Index I.I. (i.e., i50/S) of the sulphonamides was determined by plotting the % inhibition *vs* the logarithm of the inhibitor concentration. Interpolation for 50% inhibition and division of the inhibitor concentration found by the substrate concentration will give the relative affinity, expressed as the Inhibition Index, of the compound.

Materials

[7-^{14}C]PABA (spec. act. 10·1 mCi mmol^{-1}) was purchased from ICN, California. H_2-pter was prepared by reducing 2-amino-4-hydroxy-6-hydroxymethylpteridine (Thijssen, 1973b) with sodium dithionite at pH 11 and 100° (Pfleiderer & Zondler, 1966). After completion of the reduction (the pH falls gradually, during reduction, to pH 6·3), 2 drops of mercaptoethanol were added and as much of concentrated HCl to bring the pH to 2·8. The precipitated H_2-pter was centrifugated, washed 3 times with 0·1 M mercaptoethanol and lyophilized. The product was stored in a dessicator in the dark and remained unchanged for at least 1 month [E(254, 0·1N HCl)/E(230, 0·1N HCl) = 1·8].

Sulphonamides

Sulphanilamide, sulphapyridine, sulphadiazine, sulphamethazine, sulphathiazole, sulphacetamide and sulphaethylthiadiazole were of Dutch Pharmacopea quality. Sulphabenzamide was purchased from K and K Laboratories. Sulphadimethoxine, sulphamethoxazole and sulphisoxazole were gifts from La Roche. Sulphasomidine and sulphamoxole were gifts from Nordmark. Sulphanilyl-3,4-xylamide was a gift from Geigy. Sulphanilylcyanamide was prepared according to Winnek, Anderson & others (1942). N^1-phenyl-, N^1-methyl-, N^1-ethyl-, N^1N^1-dimethyl-, N^1N^1-diethyl- and N^1-ethyl-N^1-methyl-sulphonamide were prepared by standard methods (Northey, 1948).

RESULTS

Fig. 1 shows, for three different PABA concentrations, the dependence of the % inhibition of H_2-pteroate synthesis on the logarithm of the concentration of sulphathiazole. The mean Inhibition Index of sulphathiazole, obtained from six separate

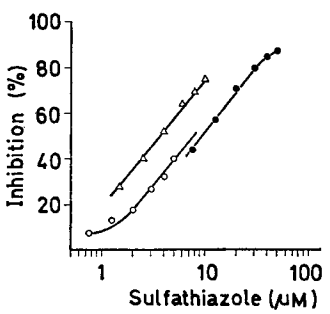

FIG. 1. Inhibition Index for sulphathiazole at different PABA concentrations. Plot of the % inhibition *vs* log of the sulphathiazole concentration used in the determination of the Inhibition Index. △—△, PABA, 10·9 μM, I.I., 0·33; ○—○ PABA, 21·9 μM, I.I., 0·37; ●—●, PABA, 28·8 μM, I.I., 0·34.

experiments, was 0·34 with a range from 0·31–0·37. The Inhibition Indices, found for the mono-substituted sulphonamides, are listed in Table 1. For some of these sulphonamides, Table 1 also gives the antibacterial activities (Minimum Inhibition Concentration) for *E. coli*, as obtained from literature (Seydel, Krüger-Thiemer & Wempe, 1960; Seydel, 1971; Seydel & Wempe, 1964, 1971).

Table 1. *Inhibition Index I.I., pKa and minimum inhibitory concentrations of some* N^1*-substituted sulphonamides.*

No.	Compound	I.I.[1]	I.I.x[2] ($\times 10^2$)	pKa	MIC[3]
1	N^1-Ethylsulphanilamide	28	3·6	10·88[4]	
2	N^1-Methylsulphanilamide	21	3·5	10·77[5]	
3	Sulphanilamide	10	3·6	10·43[5]	128[6]
4	N^1-Phenylsulphanilamide	1·90	18·2	8·97[7]	16[7]
5	Sulphapyridine	0·67	18·2	8·43[5]	4[6]
6	Sulphasomidine	0·45	36·0	7·4[6]	2[6]
7	Sulphamethazine	0·51	41·9	7·34[5]	2[6]
8	Sulphathiazole	0·34±0·02	30·1	7·12[5]	2[6]
9	Sulphamoxole	0·27	24·5	7·0[8]	3·5[9]
10	Sulphadiazine	0·78	75·7	6·48[5]	1[6]
11	Sulphadimethozine	0·27	27·0	5·9[6]	1[6]
12	Sulphamethoxazole	0·25	25·0	5·7[6]	1[6]
13	Sulphaethylthiadiazole	0·43	43·0	5·45[6]	2[6]
14	Sulphacetamide	3·5	350·0	5·38[5]	2[6]
15	Sulphanilyl-3,4-xylamide	0·55	55·0	4·95[6]	4[6]
16	Sulphisoxazole	0·46	46·0	4·9[6]	2[6]
17	Sulphabenzamide	0·95	95·0	4·57[5]	5·6[10]
18	Sulphanilylcyanamide	5·2	520·0	2·92[5]	

[1] Inhibition Indices were determined as described under Experimental section.
[2] I.I.x means Inhibition Index corrected for the fraction of the molecules ionized at pH = 8, x = Ka/([H$^+$] + Ka). [3]Minimum Inhibition Concentration in μmol litre^{-1} for *E. coli*. [4]Determined by potentiometric titration of 0·1 mmol of the sodium salt with 0·01 N HCl. [5]According to Bell & Roblin (1942). [6]According to Seydel & others (1960). [7]According to Seydel (1971.) [8]Determined according to the procedure described by Bell & Roblin (1942). [9]According to Seydel & Wempe (1971). [10]According to Seydel & Wempe (1964).

A plot of log I.I. and of log MIC *vs* pKa is shown in Fig. 2A. The theoretical curves according to Bell & Roblin (1942), Breuckner (1943) and Cowles (1942) are also given. With the exception of sulphadiazine and sulphacetamide, the logarithm of the Inhibition Index showed a parabolic relation to pKa, with an optimum between pKa 6 and 7.

To investigate the importance of ionization of the sulphonamides for the interaction with the PABA binding-site, H_2-pteroate synthesis in the presence of sulphathiazole (pKa = 7·12) was estimated at different pH values. This revealed that the increase of the concentration of ionized molecules increased the extent of inhibition of H_2-pteroate synthesis (Table 2). For instance, at pH 7·0 (42% ionization) 5 μM of sulphathiazole (PABA, 21·9 μM) inhibited the enzyme reaction to about 33%, whereas at pH 8·0 (88% ionization) 43% inhibition occurred. A twofold increase of the sulphathiazole concentration at the same extent of ionization, however, gave rise to a greater enhancement of the inhibition (e.g., at pH 8·0 and PABA 21·9 μM, 4 and 8 μM of sulphathiazole caused 34 and 50% inhibition respectively, Fig. 1).

If the ionized form of the sulphonamides alone is responsible for the activity (Breuckner & Cowles), then the Inhibition Index, corrected for the ionized fraction, should be independent of the dissociation constant. However, as can be seen from

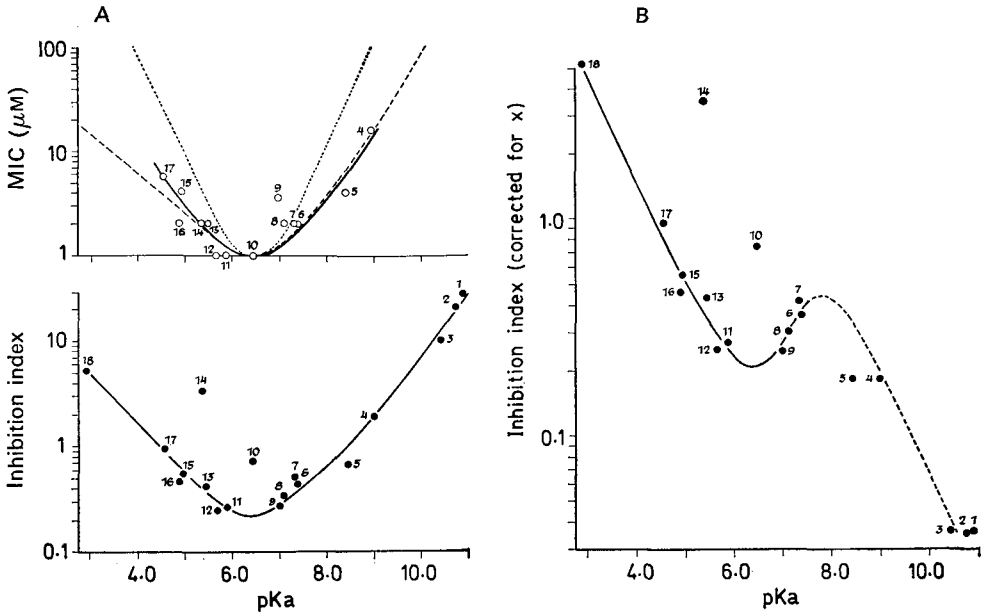

FIG. 2A. Plot of the log I.I. *vs* pKa-values of the sulphonamides, ●—●. Determination of I.I. was performed as described in experimental section and as shown in Fig. 1. ○—○, Plot of the log of the MIC (μM) of several of the sulphonamides (Table 1), together with the theoretical curves according to Breuckner (1943) and Cowles (1942), ..., and according to Bell & Roblin (1942), - - -.

B. Plot of the log I.I., corrected for the ionized fraction x, *vs* pKa [x = Ka/([H⁺] + Ka)].

these corrected values (Table 1) and from Fig. 2B, a complex relation, showing a maximum and a minimum between pKa and I.I.x (see Table 1), was found. Since it is unlikely, considering the variety in the nature of R, that the N^1-substituent of any sulphonamide directly participates in the interaction with the PABA-receptor (Bell & Roblin, 1942; Northey, 1948), the relation, as shown in Fig. 3, cannot be explained by, for instance, steric requirements of R. Hence, either a complex relation exists between the activity of the sulphonamides and their dissociation constant, or the assumption that only the ionized molecules are responsible for the activity is not correct.

Table 2. *Relation between the extent of ionization of sulphathiazole and inhibition of H_2-pteroate synthesis.*

pH*	% Ionized†	H_2-pteroate formed‡ Control	H_2-pteroate formed‡ Sulphathiazole 5 μM	% Inhibition
7·0	42	1·23	0·82	33·4
7·5	70	2·74	1·71	37·6
8·0	88	2·86	1·63	43·0
8·5	96	2·25	1·30	42·3
9·0	99	1·76	0·93	47·2

* pH at which the enzyme reaction was performed (PABA is 21·9 μM).
† The fraction of sulphathiazole ionized; x = Ka/([H⁺] + Ka).
‡ Expressed in nmol mg^{-1} of protein h^{-1}.

To see whether unionized sulphonamide molecules also interfere with the active-site of the enzyme H_2-pteroate synthetase, N^1N^1-dimethylsulphonamide, N^1-ethyl- N^1-methylsulphonamide and $N'N'$-diethylsulphonamide were examined for their inhibitory activity. All three N^1N^1-dialkyl-sulphonamides inhibited H_2-pteroate synthesis (I.I. = 14, 20, 35 respectively). Now, if it is assumed that the unionized as well as the ionized forms of the mono-substituted sulphonamides interfere with the PABA-receptor, then N^1N^1-dimethyl- and N^1-ethyl-N^1-methyl-sulphonamide are even more potent inhibitors than their mono-substituted parent compounds (Table 1 compare 2 and 1 respectively).

DISCUSSION

Since no permeability factors are involved in cell-free H_2-pteroate synthesis, the theory of Breuckner and Cowles implies, for cell-free activities, maximal activity for the sulphonamides which are completely ionized at the test conditions (pH 8·0).

The Bell and Roblin theory, on the other hand, predicts a parabolic relation between activity and pKa, since the electron density of the sulphonyl group, $-SO_2-$, will only be optimal at a particular electron-attracting power of substituent R. As is seen from Fig. 2A, with the exception of sulphadiazine and sulphacetamide, the Inhibition Indices of the sulphonamides tested show a parabolic relation to pKa.

The inhibition of H_2-pteroate synthesis by sulphathiazole at different pH values showed that increase of the degree of ionization of the inhibitor increases the inhibitory activity (Table 2). However, the enhancement of the inhibitory activity as a result of increasing ionization is less than would be expected if only the ionized molecules interfere with the active site of the enzyme. Obviously, the molecular form is also active. This can be deduced from the plot of the logarithm of I.I., corrected for the ionized fraction, *vs* pKa (Fig. 2B), and from the inhibition experiments performed with N^1N^1-dialkyl-sulphonamides. The high inhibitory activity of N^1N^1-dimethyl- and of N^1-ethyl- N^1-methyl-sulphonamide compared with N^1-methyl- and N^1-ethyl-sulphonamide respectively, may be explained in terms of the Bell and Roblin theory. Since N^1-methyl- and N^1-ethyl are very weak acids, it has to be assumed that the molecular forms are responsible for their observed inhibitory activity. The introduction of a second electron-repelling substituent on the amide nitrogen will enhance the electronegativity of the $-SO_2-$ group and, hence, will increase the affinity of the compound for the enzyme. The same consideration should hold true for N^1N^1-diethylsulphonamide, but other factors, possibly steric hindrance, may be involved.

The experimental findings presented disprove the statement that only the ionized form of sulphonamides is responsible for bacteriostatic activity as postulated by Breuckner and Cowles. Moreover, (a) the relation between the dissociation constant, i.e., the inductive effect of substituent R, and the Inhibition Index, and (b) the higher inhibitory activity of the N^1N^1-dialkylsulphonamides with respect to their mono-substituted parent compounds, are explainable by the Bell and Roblin theory. However, it remains questionable whether the electronic distribution of the $-SO_2-$ group is responsible for the observed relation, as postulated by Bell and Roblin, since no relation between any physicochemical parameter of the $-SO_2-$ group and antibacterial activity has been observed (Seydel & others, 1960; Foernzler & Martin, 1967). Moreover, some relation between the physicochemical properties of the aromatic amino-group of the sulphonamides and their antibacterial activity has been shown (Seydel & others, 1960; Seydel, 1968; Yonezawa, Muro & others, 1969). If the

electron density of the aromatic amino-group, as emphasized by these authors, is the main factor affecting activity, then obviously, an electron density on the amino-group should exist which is optimal for the drug-enzyme interactions. This would imply (regarding the parabolic relation between the activity of the sulphonamides and the inductive effect of substituent R) the existence of a non-linear relation between the inductive effect of R and the basicity of the aromatic amino-group. However, Yoshioka, Hamamoto & Kubota (1963) showed a linear decrease of the basicity of the amino-group with increasing acidity of the sulphonamide, indicating that the electron distribution of the aromatic amino-group is affected directly, via hyperconjugation, by the inductive effect of the N^1-substituent R. These findings refute a non-linear relation between the characteristics of R and of the amino-group, as proposed above. Hence, the parabolic dependency between activity and dissociation constant of the sulphonamides is not related to the physicochemical properties of the aromatic amino-group.

Foernzler & Martin (1967), from quantum chemical calculations, showed that antibacterial activity increased with increasing charge on the amide nitrogen. If this latter physicochemical property determines affinity for the enzyme, then a similar relation between structure and activity as predicted by Bell and Roblin would be expected, since the charge on the amide nitrogen would be determined by the electron-attracting and/or the electron-repelling property of the N_1-substituent, R.

For whole-cell sulphonamide activity, cell penetration, as shown by Miller & others (1972), will affect the structure-activity relation.

Acknowledgements

The author is indebted to Prof. Dr. E. J. Ariëns for valuable suggestions, and to Miss H. C. Neutelings and Mr. J. C. Martosudjono for their skilled assistance.

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