Sulfonamide Structure-Activity Relationship in a Cell-Free System. Correlation of Inhibition of Folate Synthesis with Antibacterial Activity and Physicochemical Parameters†

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Various physicochemical parameters (pK_a, Hammett σ, chemical shift, and π) of two series of substituted sulfonamides (N^1-phenyl and N^1-pyridyl) have been correlated to their inhibitory activities in a cell-free folate-synthesizing system. The rate-determining steps for sulfonamide action in the cell-free system and a whole cell system were found to have similar substituent dependencies, indicating the likelihood of a common reaction. Comparison of the linear free energy relationships obtained in the two systems indicates that the common parabolic dependency of sulfonamide antibacterial activity is best explained by the Brueckner and Cowles theory rather than the more commonly used Bell and Roblin theory. Permeability of the bacteria to these sulfonamides in the whole cell system was found to be unimportant unless they were present in the ionized form to an extent greater than 90%. The data indicate that lipophilic factors—characterized by π—are not important in the cell-free system, nor are they important in determining antibacterial *in vitro* activity when permeability is not limited by ionization.

The relationship of the chemical structure of medicinal agents to their biological activity is a fundamental problem of science. Sulfonamides have played a leading role in our current understanding of this relationship. In a pioneering paper Woods[1] recognized in 1940 that the antibacterial activities of sulfonamides were antagonized by the structurally similar compound, *p*-aminobenzoic acid (PABA). Bell and Roblin,[2] Brueckner,[3] and Cowles[4] soon realized that the antibacterial activities were related to the acid dissociation constants of the sulfonamide groups. They postulated the existence of a critical pK_a for optimal activity. Recognition of the importance of PABA in folic acid metabolism and the ability of certain folate derivatives and/or products of folate metabolism to reverse the effects of sulfonamides has led to a general theory of sulfonamide action. In principle this theory states that sulfonamides are competitive inhibitors of the enzymic incorporation of PABA into folic acid.[5,6] Brown, *et al.*,[7,8] Jaenecke and Chan,[9] Shiota, *et al.*,[10-12] and Hotchkiss, *et al.*,[13,14] have demonstrated the sequential pathway of folic acid synthesis and have shown that bacterial (or plant[15,16]) cell-free folate synthesizing extracts are inhibited by sulfonamides.

Brown[8] has determined the inhibitory activities of several sulfonamides in a cell-free folate-synthesizing system. He reported that such activities are, in general, proportional to the antibacterial activities of the compounds. The relatively small number and heterogeneity of the studied compounds have limited the utility of these activities for detailed structural-activity studies. Similarly the data have limited utility in assessing the role of permeation in antibacterial activity. This study reports the activities of a larger number of compounds belonging to two homologous series, N^1-phenylsulfonamides and N^1-pyridylsulfonamides. The antibacterial activities of these compounds have been previously evaluated in a precise and self-consistent manner.[17] These antibacterial activities have been correlated with certain physicochemical properties. Some of these compounds, however, which exist essentially in the ionized form under the test conditions, were found to have less antibacterial activity than expected on the basis of their physicochemical properties. The low activity of these compounds has been assumed to be related to their poor permeation, it being assumed that the nonionic form of sulfonamides is necessary for permeation. In order to define the role of cell-wall permeation in sulfonamide antibacterial activity several of these compounds have been further studied in the cell-free system.

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Experimental Section

Cell-Free Extracts. *Escherichia coli* mutaflor was grown on a casamino acid-dextrose medium[18] at 37.5° with aeration and shaking. Cells were harvested using a flow-through centrifuge at approximately midlog phase, washed with saline, and stored as a frozen paste. Approximately 0.8 g of cell paste per liter of culture was obtained. Extracts were prepared using modifications of the procedures of Brown[19] and Shiota.[10] Since the extracts were to be used for determination of sulfonamide activities care was taken to remove natural PABA. Cells (100 g) were ground with alumina (1:1), suspended in cold Tris·HCl buffer, pH 8 (0.01 *M*), and centrifuged to remove alumina and cellular debris. The resultant cell sap was treated with DNase (commercial sources, 2-5 mg/100 g of cells) until the viscosity decreased substantially (20 min) and then centrifuged at 100,000*g* for 1 hr to remove ribosomal material. Ribonuclease (20 mg/100 g of cells) was added to the supernatant at 37.5° for 1 hr and the resulting precipitate removed by centrifugation. Solid $(NH_4)_2SO_4$ was added to the supernatant until 85-95% saturation at 3°; after standing overnight, the precipitate was collected by centrifugation and dissolved by dialysis against Tris·HCl buffer, pH 8 (0.01 *M*). The resulting solution, after 36 hr of dialysis (3 changes of 4 l. of buffer), was placed (in 20-ml aliquots) onto a Sephadex G-25 column (1.5 × 60 cm) and eluted with phosphate buffer (K salts, pH 8, 0.0125 *M*). The initial protein eluates were combined and used as folate-synthesizing extract. The extract was kept frozen in solution in a refrigerator or was freeze-dried and kept in the refrigerator for months without change in activity. Protein was determined by the method of Folin and Lowry[20] and by the ratio of uv absorption at 280 to 260 mμ.[21] Bovine serum albumin was used as a standard.

Determination of Sulfonamide Inhibitory Activities. Single-Point Method. The concentrations of reactants in a standard reaction mixture of 400 μl were: 100 m*M* Tris·HCl, pH 7.9-8 at 20°; 10 m*M* $MgCl_2$; 50 m*M* 2-mercaptoethanol; 1 m*M* ATP; 20 μ*M* PABA; 40 μ*M* dihydropterin alcohol; sulfonamide as required; and 300 μg of extract protein. All reagents and solutions were sterile filtered prior to use. The reaction mixtures were prepared aseptically and incubated under N_2 at 37.5° for 5 hr. After this time synthesis was stopped by addition of 5 μl of 50% TCA which was then neutralized by adding 20 μl of 1 *N* NaOH. The amount of the presumed product, dihydropteroic acid,[5,9] was assayed microbiologically in terms of folate growth equivalents. For the sake of convenience the product(s) formed will be referred to simply as folate in the following sections. The ratio of the amount of folate produced in reactions containing sulfonamide to that produced in controls was used as the fractional activity, *a*. The concentration of sulfonamide causing a 50% inhibition of folate synthesis was obtained by plotting *i* (*i* =

$1 - a$) *vs.* the logarithm of sulfonamide concentration for at least 11 sulfonamide concentrations.

Kinetic Method. The composition of the reaction mixtures was the same as that in the single-point method. Reaction mixtures were stopped with TCA at various times (usually 15-min intervals). Separate reaction mixtures were prepared for each selected time. At least 7 reaction mixtures were prepared for each of 4 sulfonamide concentrations. Control reactions were run simultaneously. The amount of product was assayed microbiologically in terms of folate growth equivalents. The rate of folate synthesis was evaluated graphically from the initially linear portions of plots of 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.

Assay of Folate Growth Equivalents. The amount of folate-like product produced in a reaction mixture was determined by adding aseptically diluted aliquots of the reactions (normally 7 aliquots/reaction) to sterile folic acid assay broth (Difco) and then inoculating with *Streptococcus faecalis* ATCC 8043. Growth response was measured turbidimetrically after 16-hr incubation at 37–37.5° and the folate equivalence determined by comparison with a standard curve of response to folic acid. The range of folate used in the standard was 0.05–1 μg/ml. A new standard curve was prepared for each experiment. A more reproducible inoculum of *Streptococcus faecalis* ATCC 8043 than the usual saline suspension was prepared by allowing the organisms to grow out in assay medium containing 0.5 ng/ml of folic acid. 2-Mercaptoethanol (0.5 ml/l.) was added to the assay medium after autoclaving to prevent destruction of reduced pteridine forms. This concentration appeared effective but did not reduce the growth response to folic acid as did the use of ascorbic acid.[22] Assay broth was sterilized by membrane filtration and also autoclaved at 120° for 10 min. The optical density of the medium at 550 mμ was used to monitor the degree of dextrose degradation that had occurred during autoclaving. Only culture medium with an absorption between 0.05 and 0.15 (1-cm cuvets) was used for assay purposes. New batches of the assay medium as obtained from the manufacturer did not allow the same large growth response to folic acid and dihydropteroic acid as had older batches. The addition of pancreatic casein hydrolysate (0.5 g/l.) to the medium increased the measured growth response to a level similar to that previously obtained. The range of folic acid concentrations in which the growth of *Streptococcus faecalis* ATCC 8043 was proportional to folic acid was the same in all batches.

Minimum Inhibitory Concentrations. The methods for MIC determination have been previously described.[23-26] The MIC values are included in Table I.

Pteridines. 2-Amino-4-hydroxy-6-carboxyaldehydepteridine (PtCHO) was prepared by the method of Waller, *et al.*[27] Solutions of the aldehyde in strong alkali gave the corresponding acid (PtCOOH) and alcohol ($PtCH_2OH$).[27] The uv spectrum of PtCHO in alkali had a ratio of absorption at 360 to 260 mμ of 1.7–1.8 rather than the reported 1.4.[27] The uv spectrum of the acid and the alcohol agreed with the literature values.[11,27] The nmr spectrum of PtCHO exhibited singlets at 10.2 and 9.4 ppm as reported by Taylor.[28] PtCOOH exhibited a singlet at 9.56 ppm and $PtCH_2OH$ had singlets at 9.04 and 5.21 ppm. All spectra were measured in a Varian HA 100 nmr spectrometer with reference to TMS in CF_3COOD. Dihydropterin alcohol was prepared by reduction of the alcohol with $Na_2S_2O_4$ as described by Friedkin, *et al.*[29] Reduction was judged to be 70–80% complete by evaluation of the uv spectra.[11]

Sulfonamides. Sulfamethoxazole was the gift of the Burroughs Wellcome Co. N^1-Phenylsulfonamides and N^1-3-pyridylsulfonamides were prepared by standard methods which have been described elsewhere.[17] Their pK_a values and Hammett σ values have also been reported.[17] Chemical shift values were obtained from the component amines (anilines or 3-aminopyridines) rather than the complete sulfonamides. As reported previously[17,30] these values correlate with the pK_a values of the synthesized sulfonamides but have the advantage of being determinable before sulfonamide synthesis.

The N^1-acetyl derivative of the 2-chloro-4-nitrophenylsulfonamide was prepared following a general procedure. The N^4 primary amino group of 2-chloro-4-nitrophenylsulfonamide was protected with carbobenzoyl chloride and then acetylated with acetic anhydride at the N^1-amide after which the protecting group was removed by hydrogenation. Nmr and ir spectra were consistent with the assigned structure, mp 238–242°.

Table I. Observed Cell-Free Inhibitory Activities, Antibacterial Activities, and Physicochemical Parameters of the N^1-Phenylsulfonamides and of the N^1-3-Pyridylsulfonamides

Sulfonamides	No.	i_{50},[a] μM	k_{50},[a] μM	MIC,[b] μM	N^1 pK_a[c]	Chemical shift,[c] ppm (DMSO)	Hammett σ	Para and meta substituent values π[32]
Substituted N^1-3-pyridyl								
6-$(C_2H_5)_2$-N-	1	95.0		90.00	9.00	4.30		
6-$(CH_3)_2$-N-	**2**	80.0		32.00	8.85	4.65	−0.600[i]	
6-CH_3O-	**3**	55.0		28.80	8.37	4.72	−0.270	−0.40
2-Cl-6-CH_3O-	4	18.5		9.50	7.95	5.05	(0.230 − 0.270)	(0.59 − 0.04)
H	5	14.0		4.00	7.57	5.23	0.000	0.00
6-CH_3S-	6	12.0		5.60	7.66	5.17	−0.047	
6-Cl	**7**	6.5		1.40	7.07	5.46	0.230	0.70
2-Cl	**8**	6.5		0.70	6.76	5.52	0.230	0.59
Substituted N^1-phenyl								
4-OCH_3	**9**	75.0		34.50	9.34	4.56	−0.270	−0.04
H	**10**	45.0	≈50[d]	16.00	9.10 (8.97)[e]	4.96	0.000	0.00
4-Cl	11	35.0		13.00	8.56	5.18	0.230	0.70
4-I	**12**	25.0	20	11.25	8.17	5.24	0.276	1.26
2-Cl, 4-OCH_3	**13**	19.0		16.60	8.81	4.86	(0.23 − 0.27)	(0.59 − 0.04)
3-CF_3	14	15.0		5.60	7.98	5.48	0.450	1.07
2-Cl	15	13.5		2.80	8.18	5.26	0.230	0.59
4-$COCH_3$	16	10.5		2.00	7.52	5.99	0.874[h]	−0.37
4-CN	**17**	7.0		1.00	7.36	6.10	1.000[h]	−0.32
4-NO_2	18	7.0	5.5	1.00	6.97	6.60	1.270[h]	0.24
2-OCH_3, 4-NO_2	**19**	6.0		4.80	7.27	6.38	(1.27 − 0.27)	(−0.33 + 0.24)
2-Cl, 4-NO_2	**20**	6.0[f]	2.2	10.80	6.17	6.89	(1.27 + 0.23)	(0.59 + 0.24)
2-NO_2, 4-CF_3	**21**	5.0[f]		3	6.10[g]	7.92	(1.00 + 0.55)	
2-Br, 4-NO_2	**22**	4.0[f]	2.0	2.30	5.70	6.83	(1.27 + 0.23)	(0.75 + 0.24)
2-Cl-4-SO_2NH_2	**23**		3.0	0.85	6.51	6.05	(0.23 + 0.62)	(0.59 − 1.82[j])

[a]Cell-free inhibitory activities, i_{50} or k_{50}, were determined as described under Experimental Section. [b]Antibacterial activities have been reported previously[17,30] or were newly determined by the same method.[26] In the cases where new determinations were made the values given are the average values obtained from all the determinations. [c]The determination of the physicochemical parameters which have been used is reported elsewhere.[17,30] [d]Determination from an experiment in which only 2 concentrations of the drug were utilized (Figure 5). [e]Literature value of 9.[33] [f]Single point method values for these compounds are too high, reflecting the nonlinear kinetics observed with them, see text. [g]Extrapolated from a plot of Hammett σ constants against pK_a. [h]σ^- has been used. [i]According to Jaffé. [j]Taken from Craig.[34]

Results and Discussion

Sulfonamide Inhibitory Activities. The results of some typical single-point method experiments conducted to determine the inhibitory activities of several N^1-phenylsulfonamides are shown in Figure 1. Figure 2 shows similar data for some N^1-phenylsulfonamides. The concentration of sulfonamide causing a 50% inhibition of folate synthesis, i_{50}, was determined from such plots. The sulfonamide activities, as i_{50} values, for all the tested compounds are listed in Table I. The tested sulfonamides possessed a wide range of physicochemical properties and exhibited an approximately 20-fold variation in inhibitory activity.

Table I also gives an estimate of the antibacterial activity of these sulfonamides, the minimal inhibitory concentration —MIC—which was obtained for all the compounds in a uniform manner in a protein-free medium.[17,26] The MIC is the *minimum inhibitory concentration* necessary to prevent visible growth of *Escherichia coli* in a serial dilution test. Figure 3 is a plot of log MIC *vs.* log i_{50} or log k_{50}. It is apparent that with the exception of 5 compounds all the sulfonamides can be fitted by a single line. Log i_{50} = 0.7791 + 0.6328 log MIC, $n = 18, r = 0.9513, s = 0.0513$. It is rather surprising that a single line should fit these 18 compounds since they belong to two homologous series whose antibacterial activities show rather large differences.

This result indicates that the differences in MIC values observed are paralleled by differences in cell-free activity. Therefore these differences cannot be attributed to permeation factors but must be associated with the reaction of the sulfonamides with the extract protein. It seems most likely that permeation factors do not contribute substantially to the antibacterial activity of these 18 compounds *in vitro.*

The five compounds which do not fit this singular relationship between MIC and cell-free inhibitory values are all compounds whose pK_a values are such that under the test conditions they are at least 90% ionized. They were the only compounds studied with such high ionization. It might reasonably be expected that under such conditions the permeation of the ionized form into the cell might be the rate-determining step in the whole cell activity determinations. The Brueckner[3] and Cowles[4] theory of sulfonamide activity has indeed postulated the occurrence of such a phenomenon, *i.e.*, that it is not the extracellular ionic concentration which governs the potency of the sulfonamide but rather the intracellular ionic concentration. This intracellular ionic concentration is thought of as being limited by the permeation of unionized compound. It is assumed that only the molecular form can enter the bacterial cell. This hypothesis differs from the Bell and Roblin theory, the first time a nonlinear relationship between pK_a and activity was recognized; however, the assumption is made that the ionization is an indirect measure of the negative character of the SO_2 group which is further assumed to be correlated to activity. In the ionized form the SO_2 group is expected to be more negative and the sulfonamide therefore more active. However, it appears also that the negative character of the SO_2 group decreases with increasing acidity. The more acidic a sulfonamide, the less negative is the SO_2 group of the ionic and molecular forms and the less is the activity of both forms. It follows if the acid strength increases, bacteriostatic activity should increase until the change in the ratio of the ionized form to the molecular form is less pronounced than the decreasing activity of both forms. Both theories predict a minimum in inhibitory concentration (MIC) for a certain pK_a-pH relation, however, our experimental findings are only in agreement with the postulates of Brueckner[3] and Cowles.[4] Previous publications from our laboratory have shown that these MIC values are linearly correlated

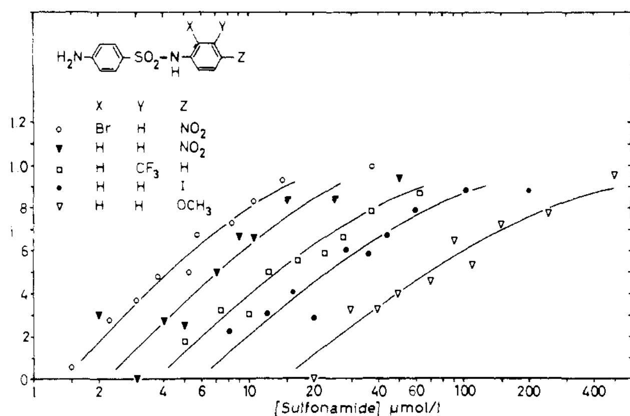

Figure 1. Determination of the inhibitory activity of several N^1-phenylsulfonamides by the single-point method. Plots of the fraction of inhibition, i, of cell-free folate synthesis *vs.* log of the sulfonamide concentration were used to estimate i_{50} values. The conditions of the experiments were as described under Experimental Section.

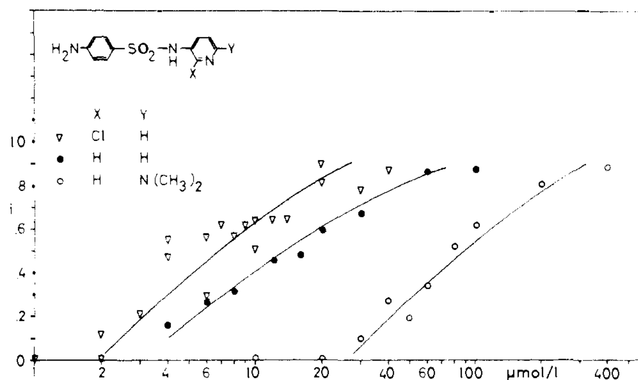

Figure 2. Determination of the inhibitory activity of several N^1-pyridylsulfonamides by the single-point method. Plots of the fraction of inhibition, i, of cell-free folate synthesis *vs.* log of the sulfonamide concentration were used to estimate i_{50} values. The conditions of the experiments were as described under Experimental Section.

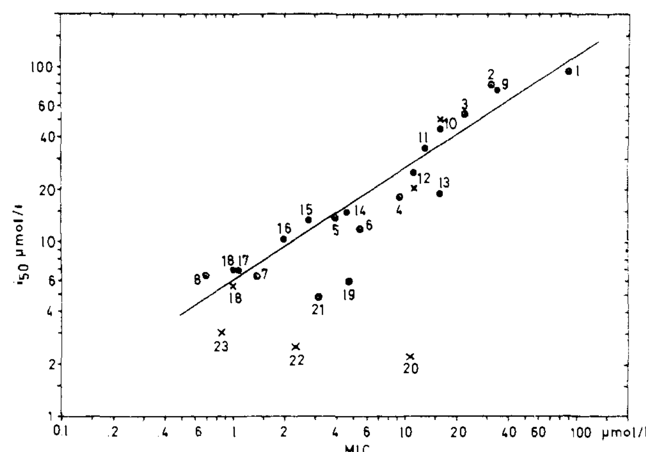

Figure 3. The relationship of cell-free inhibitory activities to whole cell inhibitory activities. A single relationship for both N^1-phenylsulfonamides (•) and N^1-pyridylsulfonamides (○) was observed. Compounds 19–23 which do not fit this relationship all have high cell-free activity and low whole cell activity, for a discussion of these compounds see the text (single-point method •), kinetic method x).

with N^1-sulfonamide pK_a values.[17,30] However, the correlations showed certain exceptions to linearity, namely compounds with very low pK_a values. Among these exceptions were the 5 compounds presently under discussion. If these compounds exhibited activities in the cell-free system related to their pK_a values, but had antibacterial properties determined, additionally, by permeation factors they would be expected to exhibit a unique relationship between MIC and cell-free activity. Indeed they all exhibit lower than expected activity in the whole cell system and very high—but not as high as expected—activity in the cell-free system. The deviation of the 5 compounds from the singular relationship of i_{50} or k_{50} values to MIC values in Figure 3 can then most probably be assigned to their poor permeation into bacterial cells, *i.e.*, a decrease in the effective fraction.

In a recent experiment, additional support for this hypothesis was obtained by testing the N^1-acetyl derivative of the 2-chloro-4-nitro-N^1-phenylsulfonamide. The MIC in whole cell systems (*E. coli*) was found to be 0.5 μmole/l., whereas the parent compound (**20**) with a pK_a of 6.8 has an MIC of 10.8. It is believed that the factor governing this difference is the ready permeation of the un-ionized acetyl derivative *vs.* the decreased permeability of the highly ionized parent compound. Inside the bacteria all the N^1-acetyl derivative would be expected to be hydrolyzed to deliver the active, ionized form. Under conditions of neutral pH and at 37° the N^1-acetyl derivatives are generally hydrolyzed completely within 6 hr and the activity of the N^1-acetyl derivative can therefore not be determined.

It should be pointed out that the occurrence of a relationship of the type seen in Figure 3 between whole cell activities and cell-free activities is good evidence that the rate-determining step in both systems is similar. While it is not definite proof that these reactions are indeed one and the same there is nothing in our data to contradict such a conclusion. The similar but nonquantitative relationship seen by Brown[8] is of course also consistent with this idea. A plot of log i_{50} *vs.* log MIC is a plot of two free energies. In a previous paper[17] it has been demonstrated that the MIC data can be considered as a reflection of the generation rate constants of the growth of *E. coli* in the presence of sulfonamides. The activity parameter k_b obtained from a kinetic approach in growing cultures[31] and the activity parameter obtained from minimum inhibition experiments are comparable $k_b/k_{b_0} = \mathrm{MIC_0/MIC_i}$. A plot of log MIC or log i_{50} *vs.* physicochemical data as Hammett σ is therefore a free energy relationship.

During the initial determinations of the cell-free inhibitory activities it was noted that several compounds gave inhibitory values less than expected, in particular those with very low N^1 pK_a values. An explanation for this behavior was found by examination of the kinetics of the inhibited reaction mixtures.

Figure 4a shows a typical kinetic method experiment in which the amount of folate-like material produced in the reaction mixtures as a function of time was determined for several sulfonamide concentrations. The concentration of sulfonamide causing a 50% inhibition in the rate of folate synthesis, k_{50}, was determined from plots such as the one in Figure 4b. The sulfonamide activities obtained, as k_{50} values, are listed in Table I. Because of the large amount of work involved in such determinations it was not possible to perform such experiments for all the available compounds. However, it seems clear that the activities of all but the very highly active compounds are the same as those obtained with the single-point method.

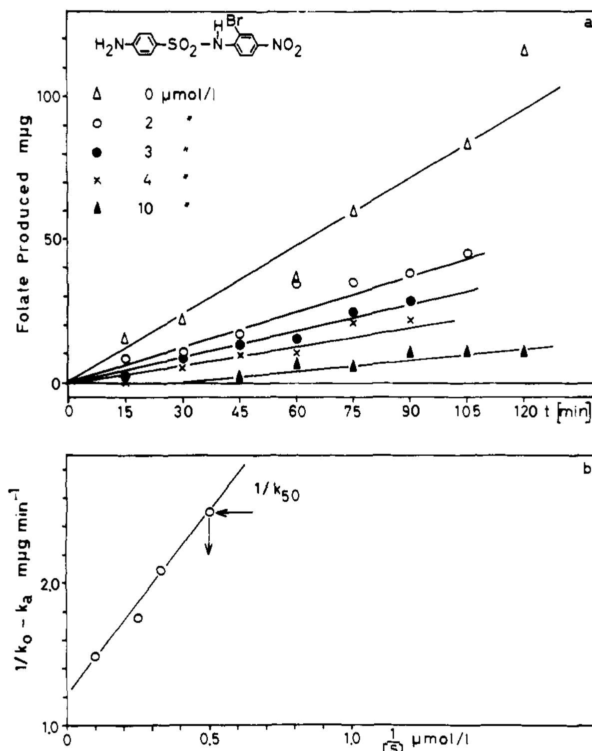

Figure 4. Determination of the inhibitory activity of 2-bromo-4-nitro-N^1-phenylsulfonamide by the kinetic method. Rate constants for folate synthesis in the presence of several concentrations of 2-bromo-4-nitro-N^1-phenylsulfonamide were obtained from kinetic experiments (a) and used in Lineweaver-Burke type plots (b) to obtain estimates of k_{50}.

An explanation for this was found when the kinetics of inhibition was studied for the length of time involved in the single-point method experiments.

Figure 5 shows that the kinetics of inhibition of a less active sulfonamide, N^1-phenylsulfonamide, are pseudo-zero-order throughout the entire time period. However, in the case of the highly active compound, 2-bromo-4-nitro-N^1-phenylsulfonamide, the reactions are only linear during the initial time period (approx 2-3 hr). After this time the reactions returned to a rate of folate synthesis similar to that seen in the control reactions. Because of this effect, the amount of folate synthesized after 5 hr in the presence of the sulfonamide was only 50% less than that in the controls despite the inhibition of the initial rate by 70%. A likely reason for such behavior is depletion of the inhibitor, presumably due to its utilization as a false substrate. Further evidence in support of this theory will be presented elsewhere. It does seem clear, however, that for the highly active sulfonamides the effect on the initial rate of folate synthesis as measured by the k_{50} values should be used. For the less active sulfonamides the k_{50} values and i_{50} values are equal and the more conveniently obtainable i_{50} values may be used.

Structure-Activity Correlations. In view of the relationship between MIC values and cell-free activities (Figure 3) we would expect all physicochemical parameters (pK_a, Hammett σ, and nmr data) which gave successful correlations of MIC data to be equally successful with cell-free activities. In addition, those exceptions to correlations of MIC values with physicochemical parameters which were attributed to permeation factors should not be exceptions in correlations with cell-free activities. This does indeed appear to be the case.

Figure 6 shows the result of plotting the log of either i_{50}

	n	r	s	s_{pK_a}	s_π	s_{π^2}	F	
$\log MIC = -3.8620 + 0.5703pK_a$	12	0.9147	0.0797					(1)
$\log i_{50} = -2.2840 + 0.4322pK_a$	14	0.9761	0.0278					(2)
$\log i_{50} = -2.9905 + 0.5465pK_a$	8	0.9778	0.0478					(3)
$\log MIC = 9.489 - 1.532$ ppm (for N^1-pyridylsulfonamides)	8	0.986	0.105					(4)
$\log MIC = 5.649 - 0.890$ ppm (for N^1-phenylsulfonamides)	25	0.976	0.040					(5)
$\log i_{50} = 1.5419 - 0.7497$(Hammett σ)	13	0.9476	0.0762					(6)
$\log i_{50} = -2.26366 + 0.42926pK_a$	14	0.9754	0.028				234.88	(7)
$\log i_{50} = -2.26351 + 0.43802pK_a + 0.03153\pi$	14	0.9764		0.028	0.046		112.46	(8)
$\log i_{50} = -2.34128 + 0.43658pK_a + 0.04061\pi^2$	14	0.9764		0.03059		0.05950	112.44	(9)
$\log i_{50} = -2.32272 + 0.43389pK_a + 0.02408\pi + 0.03096\pi^2$	14	0.9769		0.03221	0.05030	0.06491	69.78814	(10)

or k_{50} against the sulfonamide N^1 pK_a. For comparative purposes the correlation of MIC values with pK_a is also shown in eq 1 and 2; as expected, the sulfonamides showed a linear correlation between cell-free activity and pK_a. The deviations from linearity which occur in the whole cell correlation with compounds having a pK_a less than 7 do not occur in the cell-free correlation. A similar correlation (not shown in Figure 6) between pK_a and $\log i_{50}$ or $\log k_{50}$ was also found in the case of the N^1-pyridylsulfonamides (**1-8**) (Table I) (eq 3).

Close inspection of Figure 6 does reveal that two compounds with pK_a values greater than 7 do not fit this relationship as well as the other compounds do. These are both *o*-chloro-substituted compounds, *o*-chloro-*p*-methoxy-N^1-phenylsulfanilamide (**13**) and *o*-chloro-N^1-phenylsulfanilamide (**15**). The last mentioned compound is also not well correlated in the whole cell system. The reasons for this deviation are not clear. These compounds exhibit greater activity than predicted on the basis of their pK_a values or other physicochemical properties (chemical shift). As a result of the present work it is at least possible to say that the deviations of the *o*-chloro compounds noted previously[17] in the whole cell system correlations are not the result of permeation factors. Further testing of additional related com-

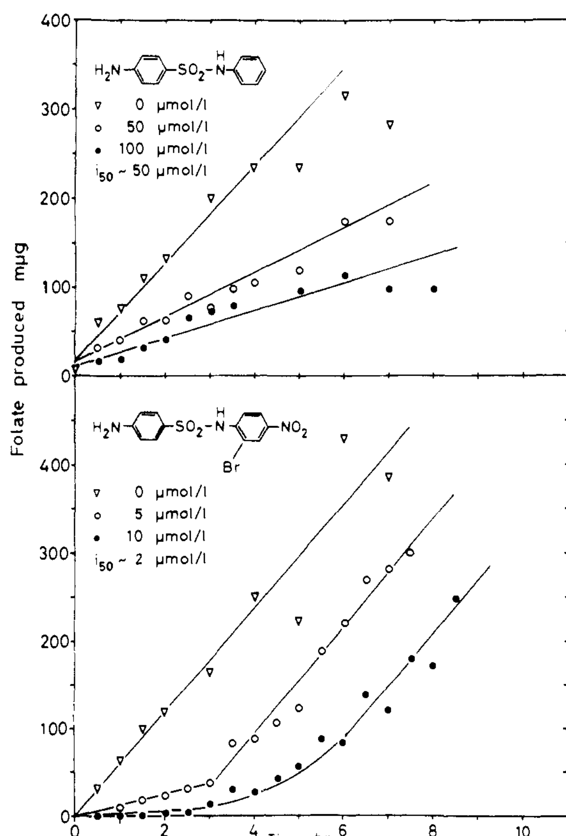

Figure 5. The kinetics of inhibition of folate synthesis by N^1-phenylsulfonamide and by 2-bromo-4-nitro-N^1-phenylsulfonamide. The kinetics of inhibition exhibited by N^1-phenylsulfonamide were found to be pseudo-zero-order throughout the experiment time. In contrast, the highly active compound, 2-bromo-4-nitro-N^1-phenylsulfonamide, only exhibited pseudo-zero-order kinetics for a short initial time. After this time the rate of folate synthesis is similar to that seen in the control.

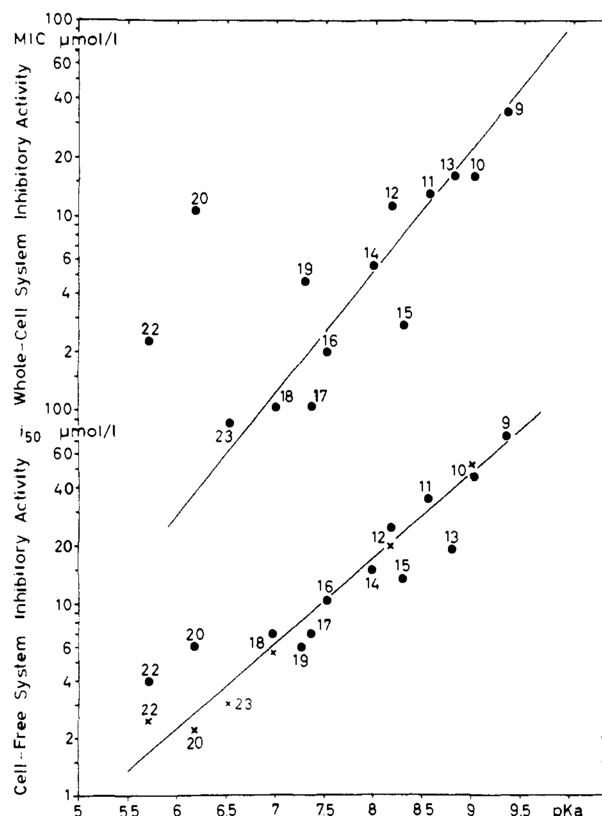

Figure 6. The relationship of inhibitory activities to N^1 pK_a values of the sulfonamides. Cell-free inhibitory activities (● single-point method, x kinetic method) of substituted N^1-phenylsulfonamides exhibited a normal linear free energy relationship with the N^1 pK_a values. A similar relationship has been previously reported[17] for whole cell activities. The line shown for this relationship is the result of all the compounds tested in this system. The points indicate only those compounds tested additionally in the cell-free system. It can be seen that the compounds with pK_a values less than approximately 7 (**20** and **22**) do not fit this linear free energy relationship.

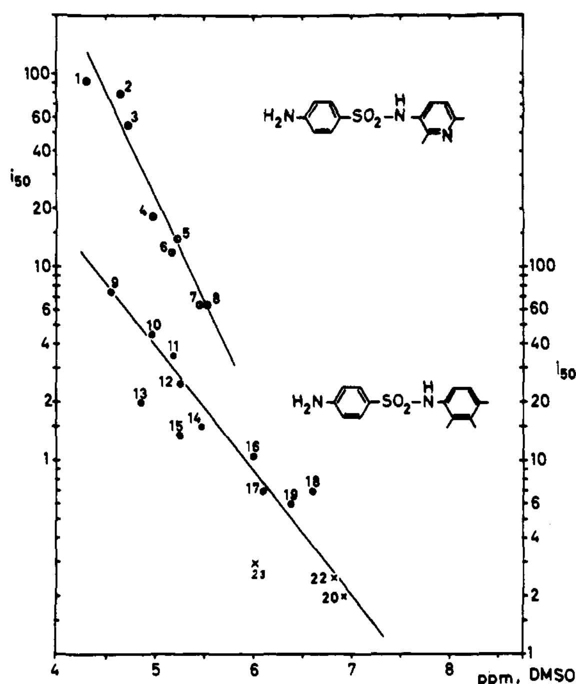

Figure 7. The relationship of cell-free inhibitory activities to chemical shift values of sulfonamide precursor amines. Chemical shift values of substituted anilines or 3-aminopyridines were obtained in DMSO using TMS as a standard. The correlation of cell-free activities with these chemical shift values is linear over the entire tested ppm scale. Previously reported correlations[17,30] of these chemical shift values with whole cell activities are not linear at higher (>6.5) ppm values, in a manner similar to the correlation of these activities with pK_a values (Figure 6). Correlation for N^1-pyridylsulfonamides: $\log i_{50} = 6.7900 - 1.0868$ ppm, $n = 8$, $r = 0.9781$, $s = 0.0944$; correlation for N^1-phenylsulfonamides: $\log i_{50} = 4.4301 - 0.5911$ ppm, $n = 14$, $r = 0.9270$, $s = 0.0690$; correlation for N^1-phenylsulfonamides (**13**, **15**, and **23** are excluded): $\log i_{50} = 4.7835 - 0.6385$ ppm, $n = 11$, $r = 0.9817$, $s = 0.0413$.

pounds is currently in progress in an effort to find the reason for this behavior.

Figure 7 shows the linear correlation obtainable with the cell-free data when nmr data are used instead of pK_a values. The nmr values employed are the chemical shifts in dimethyl sulfoxide (as ppm) of the primary amine protons of the sulfonamide precursor amines; that is: substituted anilines in the case of the N^1-phenylsulfanilamides and substituted 3-aminopyridines in the case of the pyridylsulfonamides. Both series of sulfonamides are well correlated by this type of approach. It can be seen in this figure, however, that the slopes of the lines relating activity to ppm are different for the two series. Similar results are obtained with the other physicochemical parameters and also in the whole cell correlations.[17,30]

The parallel correlation for the whole cell system[17] is given in eq 4 and 5. This difference in activity (*i.e.*, the different slopes of eq 4 and 5) between the two series must now be considered as a result of factors involved in the reaction of sulfonamides inside the cells. The exceptions to the correlation of activity with ppm seen in Figure 7 are the same *o*-chloro compounds (**13**, **15**) which were exceptions to the correlation with pK_a. It can be seen that the use of ppm values of precursor amines would enable one to predict the activity of a new sulfonamide just as well as the use of pK_a values. However, the convenience involved in predicting activity of a compound prior to synthesis makes the use of chemical shift values very attractive.

Hammett σ values, as far as they are available for the

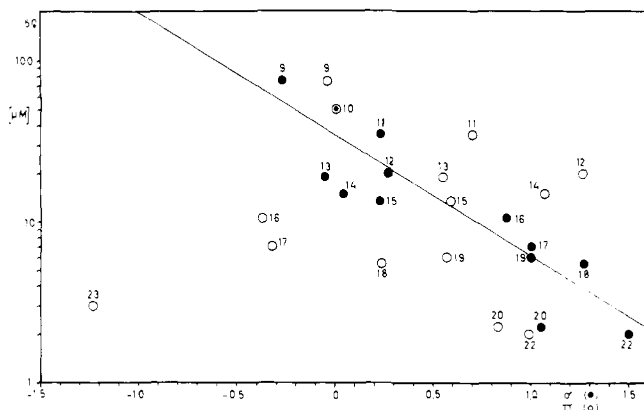

Figure 8. The relationship of cell-free activities to Hammett σ and π values. Regression analysis for correlation to Hammett σ and π:[32] $\log i_{50} = 1.5419 - 0.7497$ Hammett σ, $n = 13$, $r = 0.9476$, $s = 0.0762$; $\log i_{50} = 1.0234 + 0.0376\pi$, $n = 14$, $r = 0.0525$, $s = 0.2066$. As far as available k_{50} values are used instead of i_{50} values.

tested compounds, can also be used to obtain similar correlations. A large number of the tested compounds are disubstituted systems for which one can only use a simple addition of the individual σ values. This procedure does appear to be valid for the present compounds and a good correlation can be obtained in this manner (eq 6). However, the availability of the correlation with either pK_a values or chemical shift values would seem to be sufficient for the purpose of activity predictions.

In view of the limited role of permeation in the whole cell activity data for most of our compounds and the absence of such a factor entirely in the cell-free data it does not seem likely that partition coefficient data would further improve the correlations. This is demonstrated in Figure 8 and in the regression analysis performed in eq 7 and 8-10 where an F-test indicates no improvement in the correlation between pK_a and $\log i_{50}$ if π or π^2 is included. In any case, the correlations do not appear to be improved substantially by this type of approach. π-Substituted constants used were such as given by Fujita, *et al.*[32]

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Pharmacokinetics, Metabolism, and Urinary Excretion of [^{3}H]Alphaprodine in Dogs†

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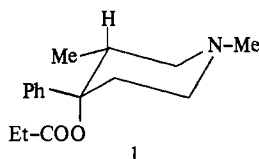
The physiologic disposition of the narcotic analgetic, alphaprodine (1), following iv adminstration in dogs conforms to a two-compartment, open-system model. The rapid disappearance of 1 from plasma and the large volume of distribution are in keeping with its rapid onset and short duration of action. The only extractable metabolite, noralphaprodine (2), achieved maximum plasma levels within 40 min after injection and remained constant for the duration (4 hr) of the experiment. The urine was found to contain 2 and conjugated metabolites whose identity presently is not known. Evidence is presented which suggests there is rapid equilibration between brain and plasma.

Alphaprodine‡ (**1**) is a potent narcotic analgetic which possesses a rapid onset and a short duration of action.[1,2] It is useful in obstetrics and surgical procedures where rapid analgesia of short duration is desired.[2] Metabolism and distribution studies in mice after sc administration suggested that the quick onset and offset of action of **1** is related to its rapid penetration into the brain and its rapid disappearance from plasma.[3,4] Investigation in man indicated that after iv injection of **1**, the plasma levels do not decline in a monoexponential fashion, but not enough data points were collected to allow conclusions regarding the nature of the pharmacokinetic model.[5] As the duration of action[6] of **1** appears to be correlated with its rapid, initial decline in the plasma,[5] it was of interest to determine the pharmacokinetic parameters in an animal model. This report describes the pharmacokinetic profile, metabolism, and urinary excretion of **1** in dogs.



Experimental Section

Materials. The syntheses of [^{3}H]alphaprodine·HCl (**1**·HCl), 1-propionyl-3-methyl-4-phenylpiperidin-4-ol (**3**), and 3-methyl-4-phenylpiperidin-4-ol (**5**) have been described previously.[4] (±)-1,3-Dimethyl-4-phenylpiperidin-4-ol (**4**) was obtained from Hoffmann-LaRoche, Inc. β-Glucuronidase, type H-2 from Helix Pomatia (Sigma Chemical Co.) containing approximately 13400 IU of β-glucuronidase and 6300 μ*M* units of arylsulfatase activity per ml, was diluted 200-fold with distilled H_2O and used for the enzymatic hydrolysis of conjugates.

Protocol. Two male mongrel dogs weighing 23.6 and 25.9 kg each received single iv doses of 2 and 1 mg/kg of [^{3}H]alphaprodine·HCl in normal saline. Heparinized blood samples were collected over the next 4 hr and the plasma was separated from red cells by centrifugation and analyzed immediately. During the first 4 hr after injection urine samples were collected through a catheter that had been inserted in the urinary bladder. The catheter was then removed, the animals placed in separate metabolic cages, and the urine collected for 24 hr. The pH values of urine samples were determined immediately after collection and the samples were frozen until analyzed.

Extraction Procedure. Plasma or urine (1 ml) was adjusted to pH > 12 with KOH, allowed to stand for 5 min, and mixed with twice its volume of C_6H_6. After shaking and centrifugation, an aliquot of the C_6H_6 phase was transferred to a centrifuge tube, mixed with 0.1 *N* HCl, shaken, and centrifuged. The C_6H_6 phase, after washing with 0.1 *N* HCl, was used for analysis of the N-demethylated metabolite. The aqueous acid phase was washed with C_6H_6, basified, and extracted with C_6H_6. The C_6H_6 phase was used for the analysis of the unchanged drug.

Acid Hydrolysis of Conjugates in Urine. Urine was extracted with benzene, neutralized with dil HCl, and mixed with 0.25 its volume of concd HCl. The sample was autoclaved (121° at 18 psi) for 80 min, adjusted to pH > 12 with KOH, and mixed with twice its volume of C_6H_6. After shaking and centrifugating, an aliquot of the C_6H_6 phase was used for analysis of conjugated metabolites.

Enzymatic Hydrolysis of Conjugates in Urine. After extraction with benzene, urine was adjusted to pH 7 with dil HCl and mixed with an equal volume of 0.1 *M* acetate buffer (pH 5.4). The mixture was shaken at 37° for 20 hr with β-glucuronidase (300 IU/ml of urine) and adjusted to pH > 12 with KOH. After shaking with twice its volume of C_6H_6, the mixture was centrifuged and an aliquot of the C_6H_6 phase was used for analysis of conjugated metabolites.

Analysis of Extracts. Radioactivity was measured in a Packard Tri-Carb Model 3375 liquid scintillation spectrometer. The C_6H_6 solutions to be analyzed were transferred to glass counting vials and mixed with 10 ml of scintillation cocktail consisting of 5.5 g of Permablend I (Packard Instrument Co.) in 1 l. of toluene. Controls with different known concentrations of the labeled drug were run concurrently with the experiment to serve as a check on the overall technique. All samples were counted for sufficient time to yield <2.5% error. The identity of the radioactive material in the benzene extracts was determined by tlc on basic alumina and ethyl acetate or $CHCl_3$–C_6H_6 (75:25 saturated with NH_4OH) as solvents.

Total Radioactivity in Urine. Urine (1 ml) was transferred into a glass counting vial, mixed with 1.5 ml of NCS (Amersham/Searle), and diluted with 10 ml of scintillation cocktail. The radioactivity in the samples was measured in a liquid scintillation spectrometer.

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‡Marketed as Nisentil by Hoffmann-LaRoche.