RESEARCH ARTICLES

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INHIBITION OF DIHYDROPTEROATE SYNTHASE FROM *ESCHERICHIA COLI* BY FIVE SULFONAMIDES

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Abstract

The inhibitory action of five sulfonamides on dihydropteroate synthase purified from ***Escherichia coli*** *was studied. The inhibitor constants (K) for sulfanilamide, 3-(4-aminophenylsulfonamido) propyl bromide, N, N′-bis (sulfanilyl)-L-cystine, sodium 3-(4-aminophenylsulfonamido) propanethiosulfate and N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine were* 4.30×10^{-4}, 7.50×10^{-4}, 3.50×10^{-4}, 5.38×10^{-4} *and* 7.50×10^{-6} *M respectively. All five sulfonamides showed their inhibitory activity by competing with* p-*aminobenzoic acid for the active site of dihydropteroate synthase.*

Introduction

Woods[1] showed for the first time that synthetic *p*-aminobenzoic acid would completely reverse the bacteriostatic activity of sulfanilamide *in vitro* against many bacteria, and postulated that sulfanilamide interferes with the utilization of p-aminobenzoic acid in enzyme systems necessary for the growth of bacteria through its similar structure. From the study of the inhibitory action of sulfonamides on enzymes in folate-synthesizing systems in bacteria[2-4], protozoa[5,6] and plants[7], it was found that some sulfonamides show their bacteriostatic activity by competing with *p*-aminobenzoic acid for the binding site on dihydropteroate synthase. Bock *et al.*[8] show that some sulfonamides can react with 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine pyrophosphate to form the dihydropterin-sulfonamide products; however, these products do not contribute significantly to the growth inhibition of *Escherichia coli* by sulfonamides[9].

Many compounds containing disulfide and thiosulfate can react with the sulfhydryl group and form the disulfide bridge at the active site of the enzyme[10-12]. Sulfanilamide disulfides strongly inhibit yeast alcohol dehydrogenase and yeast lactate dehydrogenase; the inhibition is connected with the reaction between disulfides and free sulfhydryl group of the enzyme[13]. Consequently, it is possible that the inclusion of disulfide and thiosulfate groups in sulfonamide molecule might lead to an irreversible inhibitor of dihydropteroate synthase. Thijssen[14] reports that the carboxyl group of *p*-aminobenzoic acid is necessary for the compound to interact with dihydropteroate synthase; therefore, it is likely that the carboxyl group in the side chain of N_1-substituted sulfanilamide may show high affinity toward the enzyme. In the present work, the kinetics of *Escherichia coli* dihydropteroate synthase in the presence of sulfonamides containing disulfide, thiosulfate, carboxyl and bromide groups was investigated.

Materials and Methods

Substrates and Inhibitors

[7-^{14}C] *P*-aminobenzoic acid (more than 95 % radiochemical purity, 5.7 Ci/mol) was obtained from New England Nuclear Corp. 2-Amino-4-hydroxy-6-hydroxymethylpteridine was prepared by the condensation of 6-hydroxy-2, 4, 5-triaminopyrimidine sulfate with 1, 3-dihydroxyacetone in the presence of hydrazine hydrate[15]. Its pyrophosphate ester was prepared by the coupling of pyrophosphoric acid with 2-amino-4-hydroxy-6-hydroxymethylpteridine[16]. The reduction of pteridine was carried out by the modified method described by Friedklin *et al.*[17], in which the pteridine was converted into the corresponding 7, 8-dihydro-derivative by reduction with sodium dithionite in the presence of mercaptoethanol. The concentration of 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine pyrophosphate was estimated by the method described by Shiota *et al.*[18]

N-(4-acetamidophenylsulfonyl) glycine was prepared by the condensation of 4-acetamidophenylsulfonyl chloride and glycine. N-(4-aminophenylsulfonyl) glycine was obtained from the acid hydrolysis of the corresponding acetamido-derivative. N-[4-(4-acetamidophenylsulfonamido) phenylsulfonyl] glycine was synthesized by the coupling of 4-acetamidophenylsulfonyl chloride to N-(4-aminophenylsulfonyl) glycine. N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine was obtained from the acid hydrolysis of the corresponding acetamido-derivative, its melthing point was agree with the literature value[19].

Sulfanilamide purchased from Eastman Kodak Company was recrystallized from ethanol (95 /) before use. Sodium 3-(4-aminophenylsulfonamido) propanethiosulfate and 3-(4-aminophenysulfonamido) propyl bromide were prepared by the method as described in the previous work[20].

Purification of Dihydropteroate Synthase

Dihydropteroate synthase was purified from *Escherichia coli* by a modified method of Suckling *et al.*[21] N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine

bound to Sepharose 4-B was used as the coupled gel. The spacer arm was 1, 2-bis-(3-aminopropylamino) amine. Sodium chloride (0.5 M) was present in both buffer. The enzyme was purified 209 fold. Protein was determined by Lowry's method[22].

Determination of Inhibitor Constant (K_i)

Solutions were made to contain the following components in a 250-µl volume of Tris-HCl buffer (0.48 M), pH 8.58), magnesium chloride (5 M), 2-amino-4-hydroxy-6-hydroxymethyl-7, 8-dihydropteridine pyrophosphate (6.64 mM), 2-mercaptoethanol (0.4 M), *p*-aminobenzoic acid, sulfonamide. The concentration of sulfonamide was varied in the presence of fixed concentration of *p*-aminobenzoic acid. The solution was inculated at 45°C for 10 minutes before the enzyme (4.20 µg) was added. After the reaction occurred for 10 minutes, 2-mercaptoethanol (80 µl) was added to stop the reaction. Enzyme activity was assayed by the method of Ho *et al.*[16] The amount of 7, 8-dihydropteroate was calculated from the specific activity of [7-^{14}C]*p*-aminobenzoic acid (12,870 dpm per nanomol).

The K_i values for sulfonamides were obtained from Dixon plot[23] of $\frac{1}{v}$ versus [S] where v is the initial velocity and expressed as the amount of 7, 8-dihydropteroate (micromol) per litre of assay mixture per minute, [S] is the concentration of sulfonamide and expressed as millimol per litre of assay mixture.

Results and Discussion

The Dixon plots of $\frac{1}{v}$ versus the concentration of sulfonamide in the presence of three different fixed concentrations of *p*-aminobenzoic acid were shown in Figs. 1 through 5. The K_i values for sulfanilamide, 3-(4-aminophenylsulfonamido) propyl bromide, N, N'-bis (sulfanilyl)-L-cystine, sodium 3-(4-aminophenylsulfonamido) propanethiosulfate and N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine were recorded in Table 1.

The Dixon plots indicate that all five sulfonamides showed their inhibitory activity by competing with *p*-aminobenzoic acid for the active site of dihydropteroate synthase. Sulfonamides containing the functional groups of disulfide and thiosulfate may not be the irreversible inhibitors of the enzyme since they are reversible competitive inhibitors and bind reversibly to the enzyme.

Table 1 shows that, of all five sulfonamides tested, N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine was relatively the most active competitive inhibitor since it showed the lowest K_i value. The carboxyl and two phenylsulfonyl groups might be important for the inhibitory action of this compound. 3-(4-Aminophenylsulfonamido) propyl bromide was relatively the least active inhibitor; therefore, sulfonamides containing bromide were unlikely to be the effective inhibitor of this enzyme. The inhibitory activities of sulfanilamide, N, N'-bis (sulfanilyl)-L-cystine, sodium 3-(4-aminophenylsulfonamido) propanethiosulfate were almost the same.

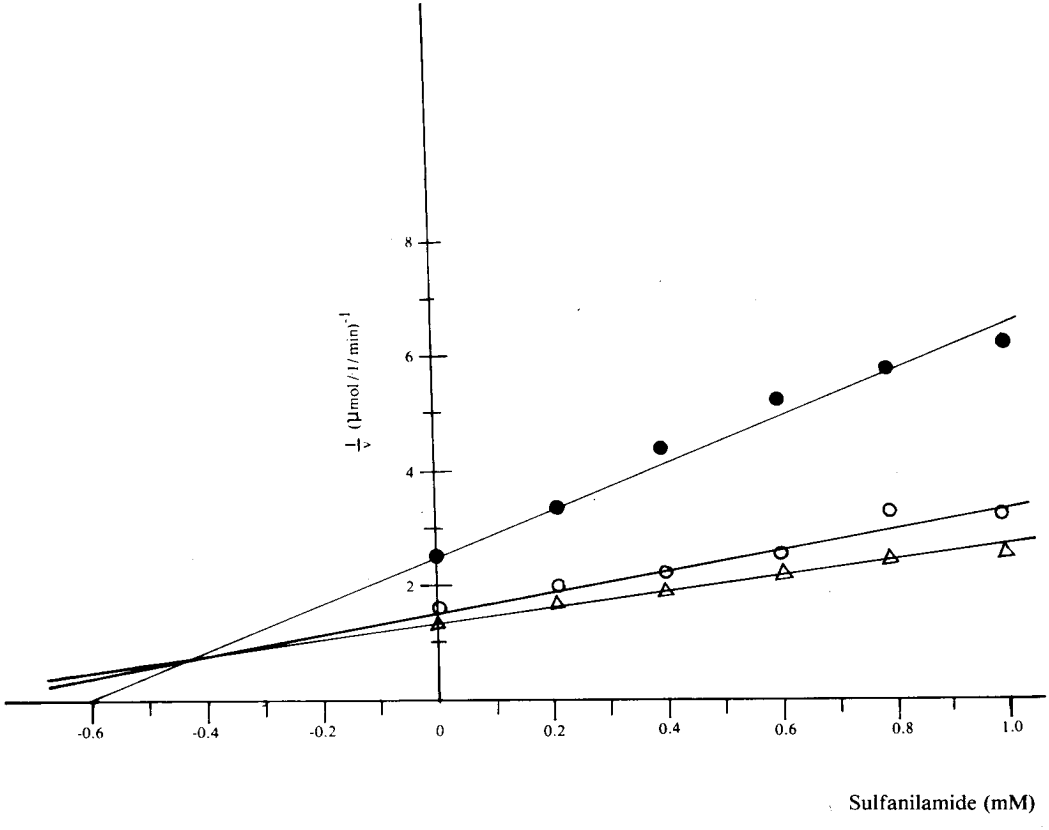

Fig. 1 Dixon plot for sulfanilamide : $\frac{1}{v}$ versus sulfanilamide in the presence of three different fixed concentrations of *p*–aminobenzoic acid (1.73×10^{-5} M (●), 3.45×10^{-5} M (○), 5.18×10^{-5} M (△)).

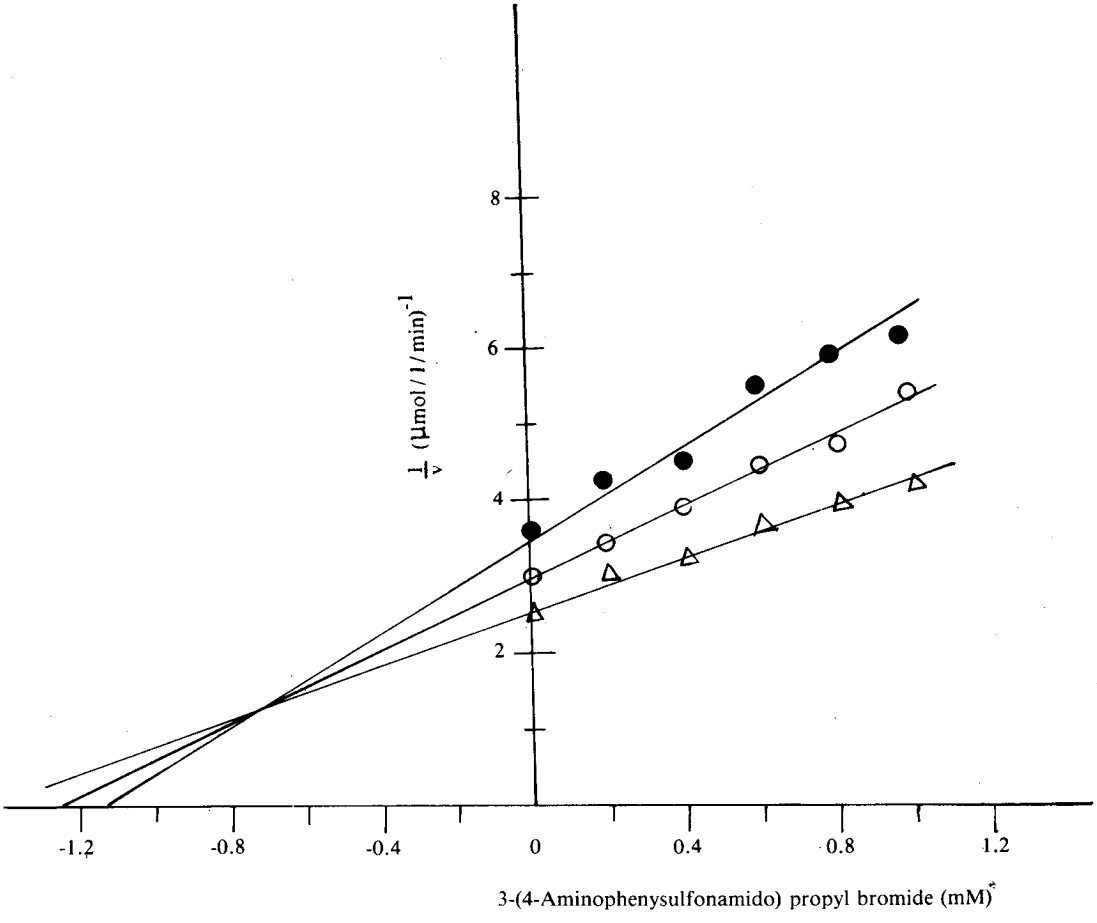

Fig. 2 Dixon plot for 3-(4-aminophenylsulfonamido) propyl bromide : $\frac{1}{v}$ versus 3-(4-aminophenylsulfonamido) propyl bromide in the presence of three different fixed concentrations of *p*-aminobenzoic acid (1.29×10^{-5} M (●), 1.73×10^{-5} M (o), 2.30×10^{-5} M (△)).

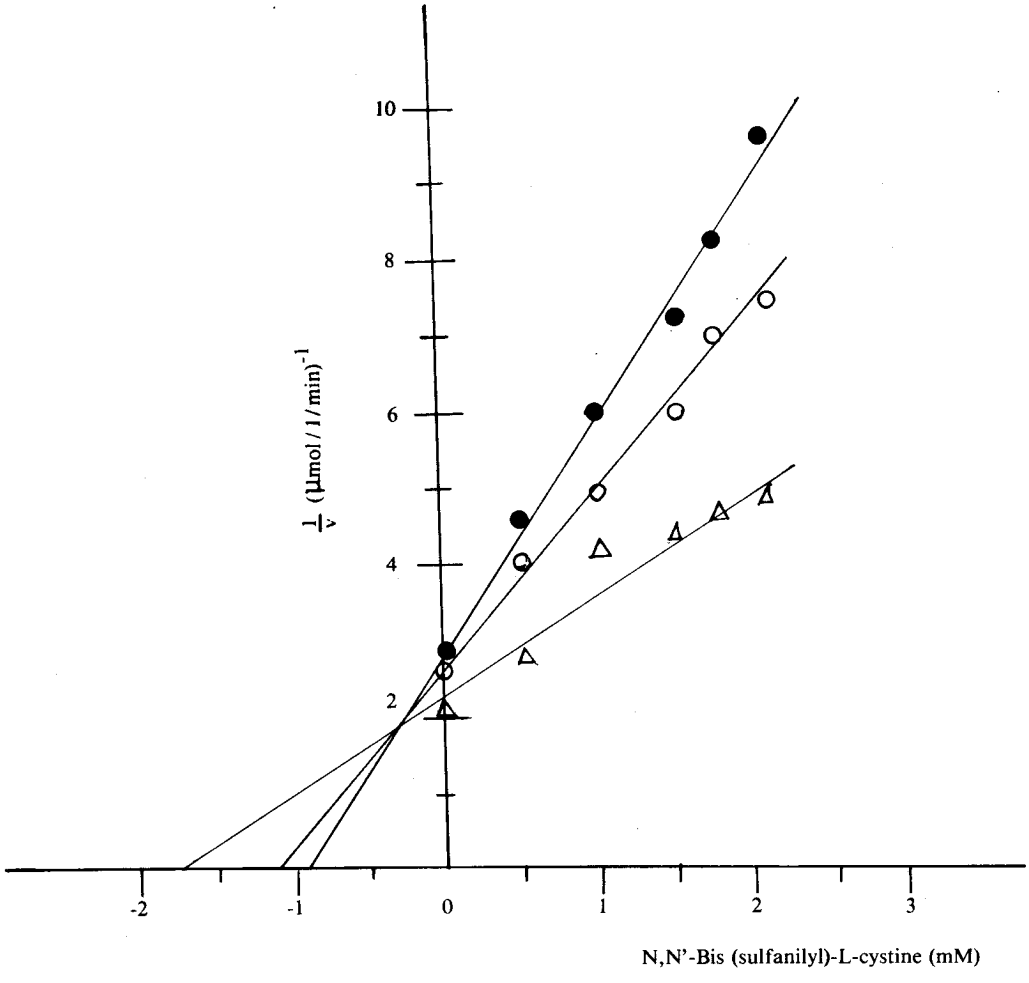

Fig. 3 Dixon plot for N, N′-bis (sulfanilyl)-L-cystine : $\frac{1}{v}$ versus N, n′-bis (sulfanilyl)-L-cystine in the presence of three different fixed concentrations of *p*-amino-benzoic acid (1.73×10^{-5} M (●), 2.30×10^{-5} M (o), 3.45×10^{-5} M (△)).

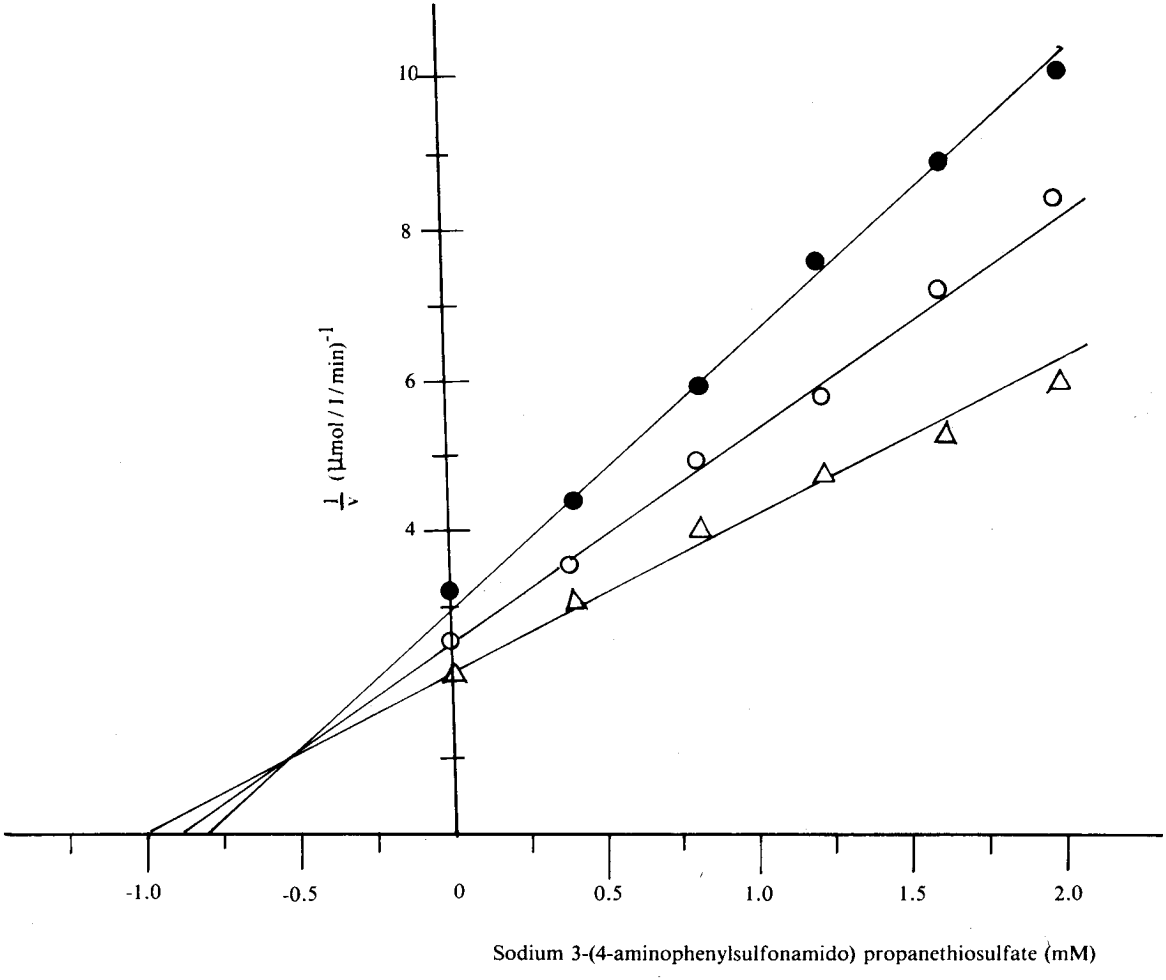

Fig. 4 Dixon plot for sodium 3-(4-aminophenylsulfonamido) propanethiosulfate : $\frac{1}{v}$ versus sodium 3-(4-aminophenylsulfonamido) propanethiosulfate in the presence of three different fixed concentrations of *p*-aminobenzoic acid (1.73×10^{-5} M (●), 2.30×10^{-5} M (o), 3.45×10^{-5} M (△)).

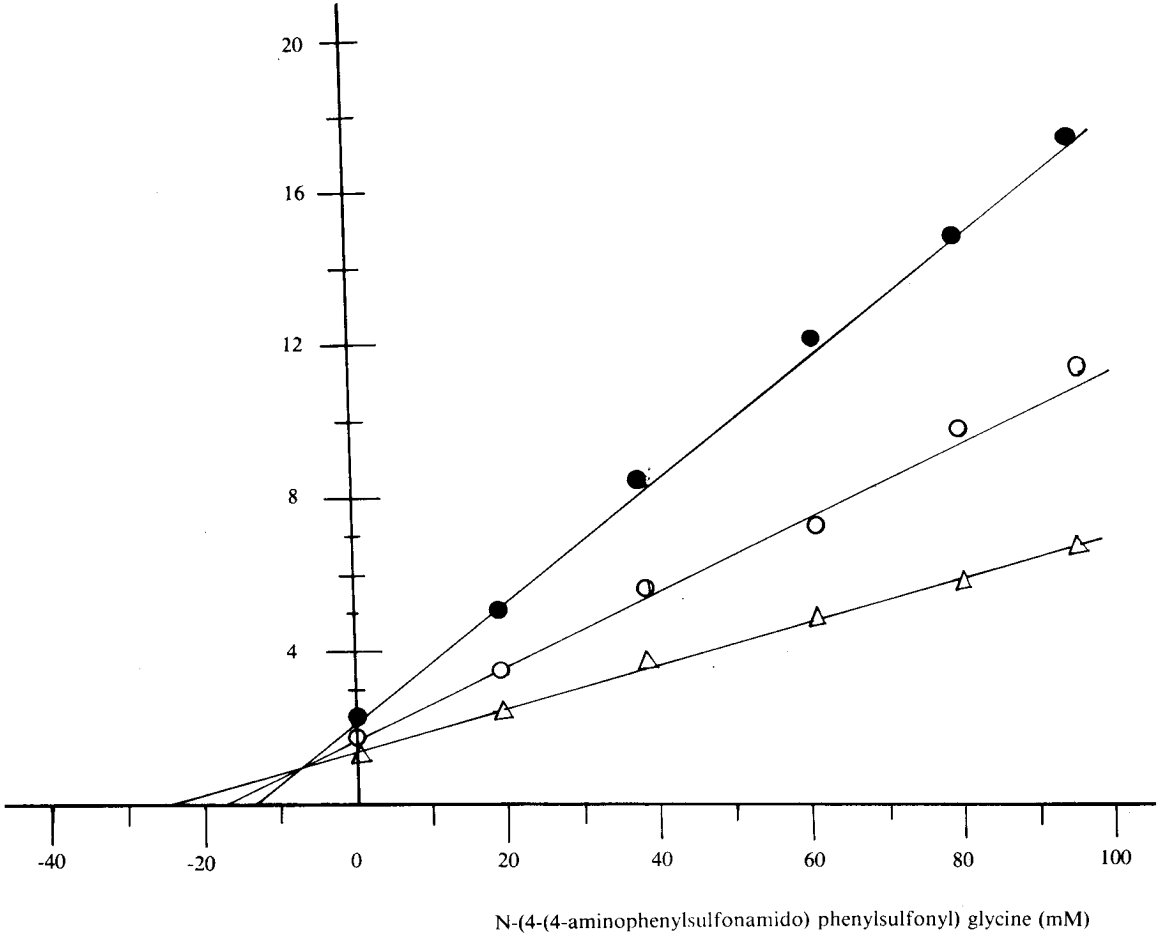

Fig. 5 Dixon plot for N-[4-(4-aminophenylsulfonamido) phenylsulfonyl glycine : $\frac{1}{v}$ versus N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine in the presence of three different fixed concentrations of p-aminobenzoic acid (5.18 x 10^{-5} M (●), 8.05 x 10^{-5} M (o), 11.50 x 10^{-5} M ( )).

Suckling *et al.*[21], using *Escherichia coli* dihydropteroate synthase, report that the K_i value for N-[4-(4-aminophenylsulfonamido) phenylsulfonyl] glycine is 8.0×10^{-7} M which is about ten times less than the value obtained from our result. This discrepancy might be due to the fact that the assay conditions for the determination of the inhibitor constant are different.

Bock *et al.*[8] demonstrate that sulfonamides are metabolized *in vitro* in the presence of dihydropteroate synthase and 7, 8-dihydropterin to sulfonamide-containing product. Roland et al. show that dihydropterin-sulfonamides were product inhibitors of dihydropteroate synthase; however, to obtain substantial inhibition of this enzyme by dihydropterin-sulfonamides *in vivo,* higher concentrations of these compounds are required than those which are attainable intracellularly. Therefore, the inhibition by dihydropterin-sulfonamides of dihydropteroate synthase is not physiologically significant[9]. The results from our study and the observation from the other investigators[2-5, 7, 9] led to the hypothesis that competition with *p*-aminobenzoic acid may be the primary mode of the action of sulfonamides. Sulfonamides reduce the rate of 7, 8-dihydropteroate synthesis; consequently, the cell growth was retarded.

TABLE 1. INHIBITOR CONSTANT (K_i) FOR SULFONAMIDES

Sulfonamide	K_i (M)
Sulfanilamide	4.30×10^{-4}
3-(4-Aminophenylsulfonamido) propyl bromide	7.50×10^{-4}
N,N'-Bis (sulfanilyl)-L-cystine	3.50×10^{-4}
Sodium 3-(4-aminophenylsulfonamido) propanethiosulfate	5.38×10^{-4}
N-[4-(4-Aminophenylsulfonamido) phenylsulfonyl] glycine	7.50×10^{-6}

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บทคัดย่อ

ศึกษาการกระทำแบบยับยั้งของสารพวกซัลโฟนาไมด์ห้าชนิดต่อไดไฮโดรพเทอโรเอต ซินเทส ที่ทำให้บริสุทธิ์จาก *Escherichia coli* ค่าคงที่ของการยับยั้ง (K_i) สำหรับซัลฟานิลาไมด์, 3 - (4 - อะมิโนฟีนิลซัลโฟนามิโด) โพรพิล ไบรไมด์, เอ็น, เอ็น - บิส (ซัลฟานิลิน) - แอล-ซิสตีน, โซเดียม 3 - (4-อะมิโนฟีนิลซัลโฟนามิโด) โพรเพนไทโอ-ซัลเฟต และเอ็น - [4 - (4 - อะมิโนฟีนิลซัลโฟนามิโด) ฟีนิลซัลโฟนิล] ไกลซีน เท่ากับ 4.30×10^{-4}, 7.50×10^{-4}, 3.50×10^{-4}, 5.38×10^{-4} และ 7.50×10^{-6} โมลาร์ ตามลำดับ สารพวกซัลโฟนาไมด์ทั้งหมดห้าชนิด แสดงการยับยั้งโดยแข่งขันกับกรดพารา - อะมิโนเบนโซอิก ในการจับที่บริเวณกระทำของไดไฮโดรพเทอโรเอตซินเทส