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Potentiating Effect of Pyrimethamine and Sulfadoxine against Dihydrofolate Reductase from Pyrimethamine-Sensitive and Pyrimethamine-Resistant *Plasmodium chabaudi*

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Dihydrofolate reductase was partially purified from a pyrimethamine-sensitive *Plasmodium chabaudi* clone and a pyrimethamine-resistant clone derived from it and used in a study of the inhibitory effect of pyrimethamine and sulfadoxine, both alone and in combination. Kinetic analysis of the inhibitory effect of sulfadoxine against the enzyme from pyrimethamine-sensitive and -resistant parasites revealed that the drug inhibited the former enzyme competitively, with an inhibition constant (K_{is}) of 0.7 ± 0.4 mM, but inhibited the latter enzyme noncompetitively, with K_{is} and K_{ii} of 8.9 ± 1.2 and 4.1 ± 1.2 mM, respectively. Previous studies also showed competitive inhibition by pyrimethamine on the former enzyme and noncompetitive inhibition on the latter enzyme, with some 200-fold-lower affinity. Sulfadoxine and pyrimethamine exhibited a mutually potentiating effect on the enzyme activity, as revealed by the concave isoboles and the fractional inhibitions of less than unity. A potentiating effect was observed for the enzymes from both sources and was not dependent on the degree of the purification of the enzyme. Our results can be explained by assuming simultaneous binding of two inhibitors on the enzyme.

Dihydrofolate reductase (5,6,7,8-tetrahydrofolate: $NADP^+$ oxidoreductase; EC 1.5.1.3), which catalyzes the NADPH-dependent reduction of dihydrofolate to tetrahydrofolate, has been regarded as one of the crucial targets for bacterial and protozoal chemotherapy. The enzyme was shown to be the molecular site of action of a number of antifolates (2, 8, 32). In malaria chemotherapy, pyrimethamine is one of the important and powerful antifolates which can be employed for antiparasitic action (20), except for the fact that resistance is easily induced (29). It has been demonstrated that the drug binds the parasite enzyme with much greater affinity than it binds the corresponding enzyme from the host (15). This observation led the investigators to propose that the basis for the successful chemotherapy of malaria is the selective action of the drug against plasmodial dihydrofolate reductase (15).

When sulfonamides, which are inhibitors of dihydropteroate synthetase (EC 2.5.1.15), were used in combination with pyrimethamine or other inhibitors of dihydrofolate reductase, the antimalarial action of the combined drugs was greatly enhanced (3, 17, 22, 38). This result is of great advantage, since the dosage of individual components required for antimalarial activity is reduced (39, 42). In addition, such potentiating effect has been reported to be remarkably effective against pyrimethamine-resistant plasmodia (23, 30, 34). Despite the wide application of these drug combinations, the mechanism of potentiation has not been well elucidated. It has generally been accepted that sequential blocking of different enzymes in the same metabolic pathway can lead to synergism (7, 18, 33). However, an observation that sulfonamides can be moderately potent inhibitors of bacterial dihydrofolate reductase from *Escherichia coli* led to an alternative hypothesis based on simultaneous inhibition of dihydrofolate reductase by both pyrimethamine and sulfonamides as a mechanism of potentiation (31). Although the observation has been disputed by other workers (1, 11, 41), the hypothesis still remains untested. Fluorometric measurements of the binding of 2,4-diaminopyrimidine and *p*-aminobenzoyl-L-glutamate to *Lactobacillus casei* cells revealed that the two fragments bind the bacterial enzyme simultaneously and cooperatively (5, 6). Moreover, a recent study concerning the in vitro evaluation of the antimalarial activity of pyrimethamine and sulfadoxine against *Plasmodium falciparum* also suggested the simultaneous-inhibition hypothesis as a possible mechanism of potentiation (13). In this paper we present the results of studies on the inhibition by pyrimethamine and sulfadoxine, alone and in combination, of partially purified dihydrofolate reductase from a clone of pyrimethamine-sensitive *P. chabaudi* and from a resistant clone derived from it. The findings that sulfadoxine can inhibit the parasite dihydrofolate reductase and that the resulting isobologram for pyrimethamine-sulfadoxine is concave, with the sum of the fractional inhibitory concentrations being <1, indicate that synergism exists at the enzyme level.

MATERIALS AND METHODS

Parasites. The pyrimethamine-sensitive *P. chabaudi* clone (strain AS) and its resistant derivative [AS(Pr_1)], which is resistant to 15 mg of pyrimethamine kg^{-1} (26), were kindly provided by D. Walliker, University of Edinburgh. The parasites were maintained in our laboratory by weekly intraperitoneal inoculation of infected blood into Swiss albino mice (weight, 30 to 35 g each) supplied by the National Animal Centre, Salaya Campus, Mahidol University. The infected mice were kept in a room which was illuminated between 5:30 p.m. and 8:30 a.m. (25). The mice were continuously fed with water supplemented with 0.01% *p*-aminobenzoic acid.

Preparation of dihydrofolate reductase from the parasites. On day 5 of infection the mice were sacrificed, and blood was collected by cardiac puncture with acid-citrated dextrose as anticoagulant (0.15 ml/ml of blood). Plasma and buffy coat were removed by centrifugation (2,000 × *g* for 10 min). The infected erythrocytes were suspended in an equal

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volume of cold phosphate-buffered saline (PBS; pH 7.4) and passed through a column of cellulose CF-11 for further removal of leukocytes (35). The erythrocytes were washed an additional three times with cold PBS (pH 7.4). After being washed, the packed erythrocytes were suspended in an equal volume of 0.15% saponin in PBS (pH 7.4). The suspension was mixed and incubated at 37°C, with shaking, for exactly 20 min. Immediately after incubation, 2 to 3 volumes of cold PBS (pH 7.4) were added, and the suspension was centrifuged at 2,800 × *g* for 20 min at 4°C. The dark-red supernatant was discarded, and the pellet of freed parasites was washed four times with cold PBS (pH 7.4).

To prepare the enzyme from the parasites, an approximately equal volume of cold 0.01 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol was added to the washed parasites. The suspension was equilibrated at 4°C for 30 min with oxygen-free nitrogen at 1,000 lb in^{-2} in a Parr cell disruption bomb. The sample released from the disruption bomb after equilibration was subjected to centrifugation at 26,800 × *g* for 1 h at 4°C. The resulting supernatant was then transferred to dialysis tubing and concentrated at 4°C with Aquacide IIA (Calbiochem-Behring). This concentrated sample was centrifuged again at the same speed for 1 h and used for further purification.

Dihydrofolate reductase was partially purified on a Sephadex G-200 column. The crude enzyme prepared as described above was applied to a column of Sephadex G-200 (2.6 by 73.7 cm) which had been equilibrated in advance with 0.01 M sodium phosphate buffer (pH 7.4) containing 1 mM EDTA and 1 mM dithiothreitol. The column was eluted with the same buffer at a rate of 30 ml h^{-1}. Fractions of 5 ml were collected. The fractions containing enzyme activity were pooled and concentrated with Aquacide IIA.

In the experiments in which different degrees of enzyme purity were required, the enzyme was further purified on a DEAE-cellulose column. The detailed procedures will be described elsewhere.

Assay for dihydrofolate reductase activity. The activity of dihydrofolate reductase was assayed spectrophotometrically by measurement of the decrease in A_{340} as a result of NADPH utilization. The reaction mixture (1 ml) contained 0.1 M sodium phosphate buffer (pH 7.4), 100 μM 2-mercaptoethanol, 50 μM NADPH, 100 μM dihydrofolate, and enzyme. All the components except dihydrofolate (0.95 ml) were preincubated together at 37°C for 5 min. The reaction was initiated by the addition of 50 μl of 2 mM dihydrofolate. The decrease in A_{340} was monitored by a Shimadzu recording spectrophotometer UV 240. The blank sample consisted of all the reaction components except the enzyme. The specific activity of the enzyme was calculated from the combined decrease in A_{340} for NADPH and dihydrofolate by using the molar extinction coefficient of 12,300 M^{-1} cm^{-1} at 340 nm (19). Dihydrofolate was prepared by the procedure of Futterman (16). Protein was determined by the method described by Lowry et al. (24), with bovine serum albumin as standard, or by A_{280}.

Inhibition of dihydrofolate reductase by pyrimethamine and sulfadoxine. The inhibition by pyrimethamine and sulfadoxine of the enzyme from both drug-sensitive and -resistant parasites was investigated by assaying the activity of dihydrofolate reductase at various substrate concentrations in the presence of several fixed concentrations of pyrimethamine or sulfadoxine. The primary plots were then made from the reciprocal activities versus the reciprocal substrate concentrations. The straight lines were drawn according to the values of slopes or intercepts, or both, calculated from the linear-regression program with a model 3500 Perkin-Elmer Data Station. The secondary plots of slopes or intercepts, or both, (obtained from the primary plots) against the inhibitor concentrations were made, and the inhibition constants (K_{is} and K_{ii}) were calculated from the abscissa intercepts of the secondary plots by using the program described above. All the straight lines were fitted with a linear-regression program with a correlation coefficient of >0.95.

Assessment of the inhibitory effect against dihydrofolate reductase of a combination of pyrimethamine and sulfadoxine. The activities of partially purified dihydrofolate reductase from both pyrimethamine-sensitive and -resistant parasites were assayed, as described above, in the presence of both a single drug and a combination of two drugs and were expressed as a percentage of the activity of the uninhibited enzyme. The 50% inhibitory concentrations (IC_{50}s) were determined graphically by the procedure described by Peters (28). The effect of the drug combination was then assessed by constructing the isobologram. The values of the sum of the fractional inhibitions at 50% inhibition (the sum of the fractions of the concentrations which inhibit 50% of the enzyme activity when the drug is used alone) were either estimated by the method of Berenbaum (4) or calculated from the median effect principle described by Chou and Talalay (12).

Analysis of in vivo inhibitory effect with a combination of pyrimethamine and sulfadoxine. The procedure used for analyzing the combined effect of pyrimethamine and sulfadoxine in vivo was as described by Peters (27).

RESULTS

Inhibition of dihydrofolate reductase by pyrimethamine and sulfadoxine. Figure 1 illustrates the inhibitory effects of sulfadoxine against the enzyme from both sources. Sulfadoxine competitively inhibits the enzyme from the drug-sensitive parasite, with K_{is} of 0.7 ± 0.4 mM (Fig. 1A), whereas it noncompetitively inhibits the enzyme from the drug-resistant parasite, with K_{is} of 8.9 ± 1.2 mM and K_{ii} of 4.1 ± 1.2 mM (Fig. 1B). Inhibition of the enzymes by pyrimethamine has been reported in our previous studies (40): pyrimethamine competitively inhibits the enzyme from the drug-sensitive parasite, with K_{is} of 0.26 ± 0.1 mM, and noncompetitively inhibits the enzyme from the drug-resistant parasite, with K_{is} of 49.6 ± 6.7 nM and K_{ii} of 32.5 ± 12.2 nM. By assaying the enzyme activity in the presence of various concentrations of pyrimethamine and sulfadoxine, we estimated the IC_{50}s of the pyrimethamine to be approximately 1.2 and 94.0 nM for the enzyme from drug-sensitive and -resistant parasites, respectively, whereas the IC_{50}s for sulfadoxine are approximately 2.8 mM for the enzyme from drug-sensitive parasites and 13.0 mM for the enzyme from drug-resistant parasites (Table 1). A summary of the inhibition by both drugs against the enzyme from drug-sensitive and -resistant parasites is shown in Table 1.

Effect of pyrimethamine and sulfadoxine in combination on dihydrofolate reductase activity. With the demonstration that sulfadoxine inhibits dihydrofolate reductase (Fig. 1), it is of considerable interest to investigate the inhibitory effect exerted by a combination of pyrimethamine and sulfadoxine. Measurement of the enzyme activities in the presence of various concentrations of the two drugs permitted us to estimate the IC_{50}s of the drugs.

From the estimated IC_{50}s, isoboles were obtained for the enzyme from both drug-sensitive and -resistant parasites (Fig. 2A and B, respectively). The obtained isoboles were

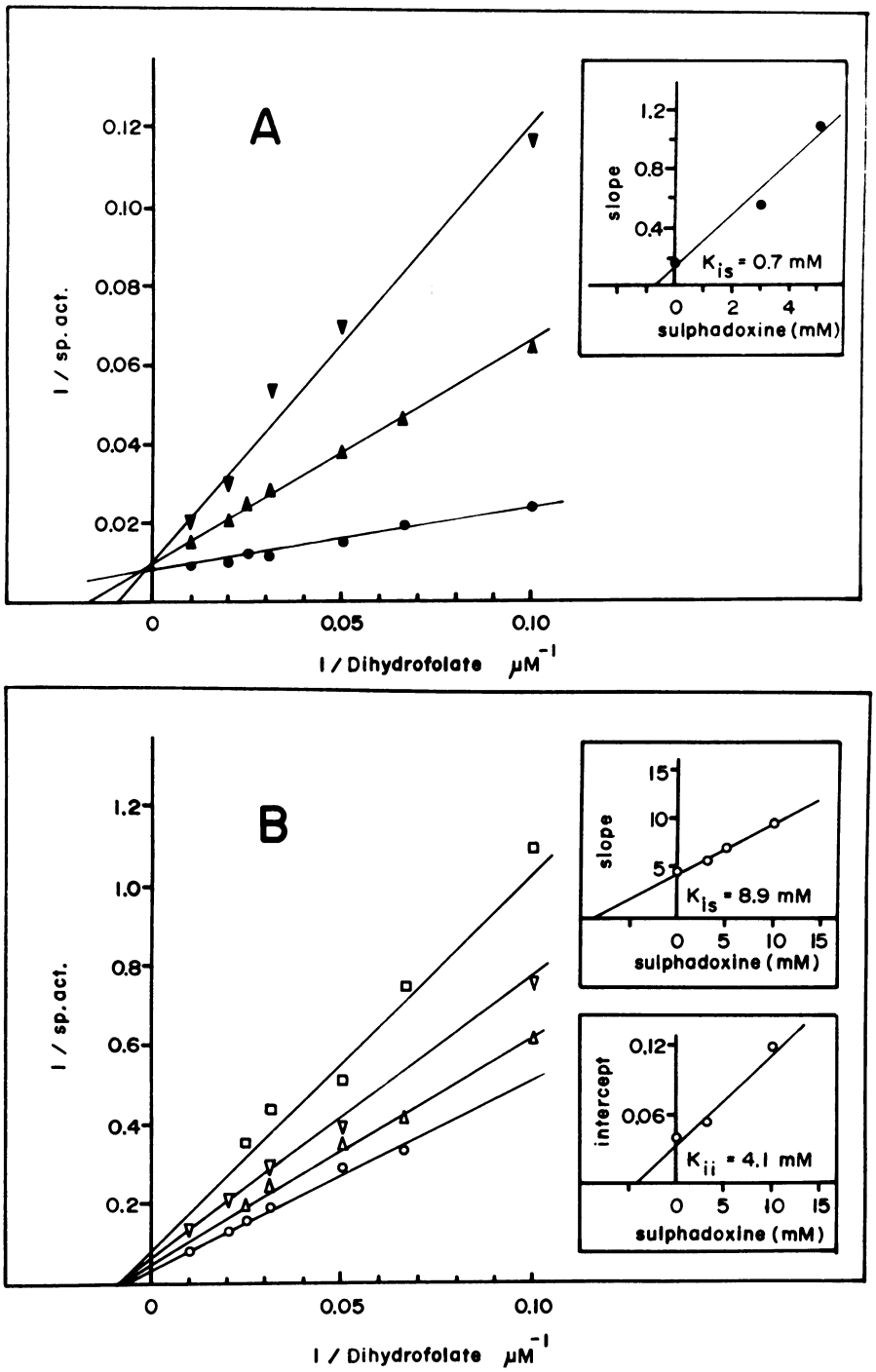

FIG. 1. Lineweaver-Burk plots of the inhibition of plasmodial dihydrofolate reductase by sulfadoxine. Enzyme activity was measured as described in Materials and Methods. To determine the inhibition constant of the drug, the enzyme was incubated with NADPH and various concentrations of sulfadoxine in the assay buffer (pH 7.4) for 5 min at 37°C. The reaction was initiated by the addition of dihydrofolate. (A) Pyrimethamine-sensitive *P. chabaudi*: ●, no drug; ▲, 3 mM sulfadoxine; ▼, 5 mM sulfadoxine. (B) Pyrimethamine-resistant *P. chabaudi*: ○, no drug; △, 3 mM sulfadoxine; ▽, 5 mM sulfadoxine; □, 10 mM sulfadoxine. Insets show the plots of the slope or intercept from a double-reciprocal plot against sulfadoxine concentrations.

concave, indicating that the combination gave rise to a potentiating effect.

Analysis of the combined effects of the two drugs on dihydrofolate reductase activity by the method of Berenbaum (4) revealed that the sum of fractional inhibitions for the enzyme from drug-sensitive and -resistant parasites is 0.62 and 0.71, respectively (Table 2). Calculation of the fractional inhibition based on the equation of median effect principle (12) also yielded comparative values of 0.66 and 0.70 for the enzyme from drug-sensitive and -resistant parasites, respectively (Table 2).

Treatment of *P. chabaudi*-infected mice with a combina-

TABLE 1. Comparison of effects of pyrimethamine and sulfadoxine against partially purified dihydrofolate reductase from pyrimethamine-sensitive and -resistant *P. chabaudi*

Source of enzyme	Drug	Type of inhibition	K_i (M)	IC_{50} (M)[a]
P. chabaudi AS(SENS)	Pyrimethamine	Competitive	$(0.26 \pm 0.1) \times 10^{-9}$ (K_{is})[b]	1.2×10^{-9}
P. chabaudi AS(SENS)	Sulfadoxine	Competitive	$(0.7 \pm 0.4) \times 10^{-3}$ (K_{is})	$(2.8 \pm 0.3) \times 10^{-3}$
P. chabaudi AS(Pr_1)	Pyrimethamine	Noncompetitive	$(49.6 \pm 6.7) \times 10^{-9}$ (K_{is})[b] $(32.5 \pm 12.2) \times 10^{-9}$ (K_{ii})[b]	$(93.5 \pm 6.5) \times 10^{-9}$
P. chabaudi AS(Pr_1)	Sulfadoxine	Noncompetitive	$(8.9 \pm 1.2) \times 10^{-3}$ (K_{is}) $(4.1 \pm 1.2) \times 10^{-3}$ (K_{ii})	$(13.3 \pm 0.7) \times 10^{-3}$

[a] Each value represents mean ± standard deviation from two experiments.
[b] Data from reference 40.

tion of the drugs also resulted in marked potentiation (Fig. 3). However, a much stronger potentiating effect was observed for in vivo combination. The fractional inhibition estimated by the method of Berenbaum (4) was 0.25, whereas the value obtained by calculation from the median effect principle (12) was 0.24.

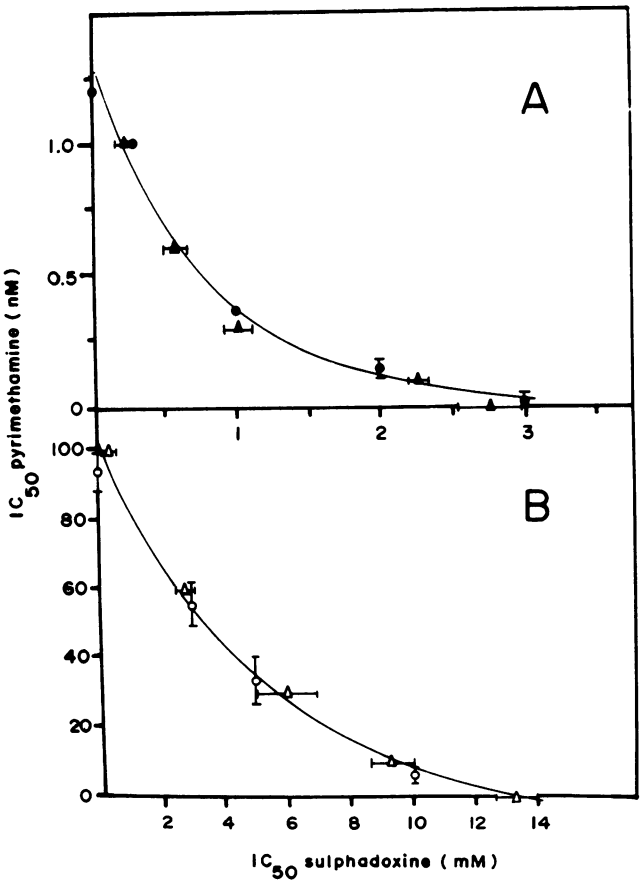

FIG. 2. Potentiating effect of pyrimethamine and sulfadoxine on *P. chabaudi* dihydrofolate reductase. The activity of dihydrofolate reductase was determined as described in Materials and Methods in the presence of various concentrations of pyrimethamine and sulfadoxine. The IC_{50}s were estimated, and isobolograms were constructed from the data in Table 2. Each IC_{50} point was the average value calculated from two experimental data. (A) Pyrimethamine-sensitive *P. chabaudi*; (B) pyrimethamine-resistant *P. chabaudi*. Symbols: ● or ○, IC_{50} of pyrimethamine when used alone or with different fixed concentrations of sulfadoxine; ▲ or △, IC_{50} of sulfadoxine when used with different fixed concentrations of pyrimethamine.

To show that the potentiating effect observed was not dependent on the degree of enzyme purification, we used different enzyme preparations for comparison: the crude enzyme (specific activity, 6.03 nmol min^{-1} mg^{-1}), the enzyme from the Sephadex G-200 column (specific activity, 17.02 nmol min^{-1} mg^{-1}), and the enzyme from the DEAE-cellulose column (specific activity, 32.62 nmol min^{-1} mg^{-1}). The potentiating effects of pyrimethamine and sulfadoxine against dihydrofolate reductases of different purity were analyzed and are shown in Fig. 4. The fractional inhibitions estimated by the method of Berenbaum (4) for the crude enzyme, the enzyme from Sephadex G-200, and the enzyme from DEAE-cellulose column were 0.54, 0.64, and 0.59, respectively.

DISCUSSION

It has been well documented that sulfonamides are inhibitors of the enzyme dihydropteroate synthetase (EC 2.5.1.15) (10). The mechanism of action was demonstrated to be the competition with the substrate *p*-aminobenzoate for binding the enzyme (36). Some sulfonamides were also shown to be alternative substrates of the enzyme dihydropteroate synthetase (37). We found that sulfadoxine, one of the sulfonamides widely used for the treatment of malaria, inhibited plasmodial dihydrofolate reductase, although the concentrations which affected the enzyme activity are some 10-fold higher than the therapeutic range (9). Similar to the inhibition by pyrimethamine (40), sulfadoxine was shown to competitively inhibit the enzyme from drug-sensitive parasites and to inhibit noncompetitively the en-

TABLE 2. Comparison of the estimated and calculated fractional inhibitions of pyrimethamine and sulfadoxine in combination and effect of drug combination on dihydrofolate reductase activity and growth of parasite

Source	Fractional inhibition	
	Estimated[a]	Calculated[b]
Dihydrofolate reductase from *P. chabaudi* AS(SENS)	0.62	0.66
Dihydrofolate reductase from *P. chabaudi* AS(Pr_1)	0.71	0.70
Parasitemia of *P. chabaudi*-infected mice[c]	0.25	0.24

[a] The values were estimated by the method of Berenbaum (4).
[b] The calculation was made according to the equation described by Chou and Talalay (12) at the point at which the combination of two drugs gives the most marked potentiation, i.e., ca. 0.38 nM pyrimethamine and ca. 0.94 mM sulfadoxine for the drug-sensitive enzyme; ca. 33.8 nM pyrimethamine and ca. 4.6 mM sulfadoxine for the drug-resistant enzyme.
[c] The values were estimated and calculated from Fig. 3. The point at which the combination of the two drugs gives the most potentiating effect is 0.0067 mg of pyrimethamine kg^{-1} and 0.24 mg of sulfadoxine kg^{-1}.

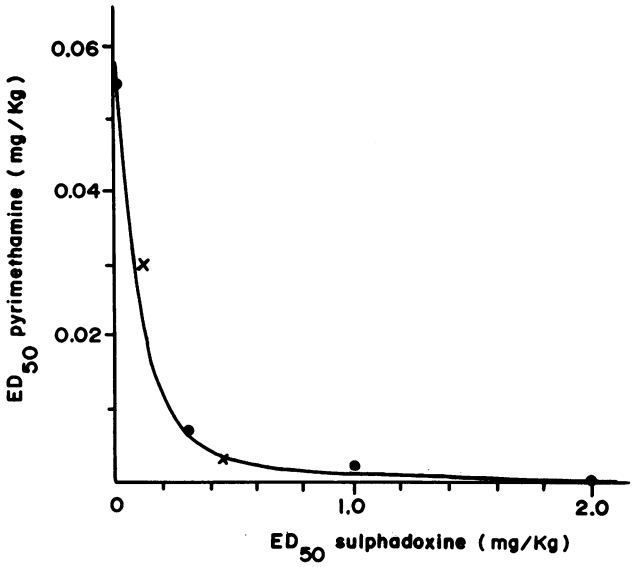

FIG. 3. Potentiating effect of pyrimethamine and sulfadoxine on mice infected with pyrimethamine-sensitive *P. chabaudi*. Groups of mice were treated with various combinations of pyrimethamine and sulfadoxine for four successive days, as described in Materials and Methods. Parasitemia was determined from thin blood films made on day 5. The 50% effective doses (ED_{50}) were estimated, and the isobolograms were constructed as in Fig. 2. Symbols: ●, 50% effective dose of pyrimethamine when used alone or with different fixed concentrations (mg/kg of body weight) of sulfadoxine; ×, 50% effective dose of sulfadoxine when used alone or with different fixed concentrations of pyrimethamine.

zyme from drug-resistant parasites (Fig. 1). The K_is and IC_{50}s for sulfadoxine are 10^5- to 10^6-fold higher than those reported for pyrimethamine (Table 1). It is also noteworthy that, while the K_{is} of pyrimethamine for the enzyme from pyrimethamine-sensitive parasites is 191-fold lower than the corresponding K_{is} for resistant parasites, the K_{is} of sulfadoxine for the former is only 13-fold lower. Hence, a decrease in affinity for binding with pyrimethamine as a result of alteration in the structure of the enzyme from drug-resistant parasites (40) could also cause a decrease in affinity for binding to sulfadoxine, although to a much smaller extent.

The inhibitory effect of pyrimethamine plus sulfadoxine on the activity of plasmodial dihydrofolate reductase was assessed by constructing the isobolograms. The enzymes from both drug-sensitive and -resistant parasites exhibit concave isoboles with fractional inhibitions of less than unity, the conditions which fulfill the criteria of potentiation. It can be noted from the isobolograms (Fig. 2) that, for example, for the enzyme from drug-sensitive parasites, only 0.38 nM pyrimethamine and 0.94 mM sulfadoxine were required to inhibit 50% of enzyme activity, while 1.2 nM pyrimethamine or 2.8 mM sulfadoxine was needed to produce the same effect if the drugs were used separately. The same phenomenon was observed for the enzyme from drug-resistant parasites, except that much higher concentrations were needed: a combination of 33.8 nM pyrimethamine with 4.6 mM sulfadoxine could inhibit 50% of the enzyme activity, while 94.0 nM pyrimethamine and 13.0 mM sulfadoxine were needed to produce the same inhibitory effect individually. It is surprising that, despite the different inhibitory patterns of pyrimethamine and sulfadoxine observed for the enzyme from drug-sensitive and -resistant parasites, there was only a small difference in the potentiating effect observed between the two enzymes, since the fractional inhibitions (both estimated and calculated) for the enzyme from both sources are comparable (Table 2).

The potentiating activity of pyrimethamine and sulfadoxine was similar, irrespective of the degree of purification of dihydrofolate reductase (Fig. 4). This makes unlikely the possibility that competition for binding with dihydrofolate reductase and other contaminants in the preparation of the two drugs is responsible for the effect observed. It is likely that potentiation occurs as the result of simultaneous binding of the two drugs on the enzyme molecule, as theoretically shown by Webb (43).

The potentiating effect has been demonstrated in vivo in mice infected with drug-sensitive *P. chabaudi* organisms (Fig. 3). The results obtained are similar to those reported when various combinations of antifols and sulfonamides were used (14, 22, 28). The estimated and calculated fractional inhibitions of approximately 0.25 (Table 2) imply that in vivo effect of drug combination is much more potent than the effect on dihydrofolate reductase activity. Although it is

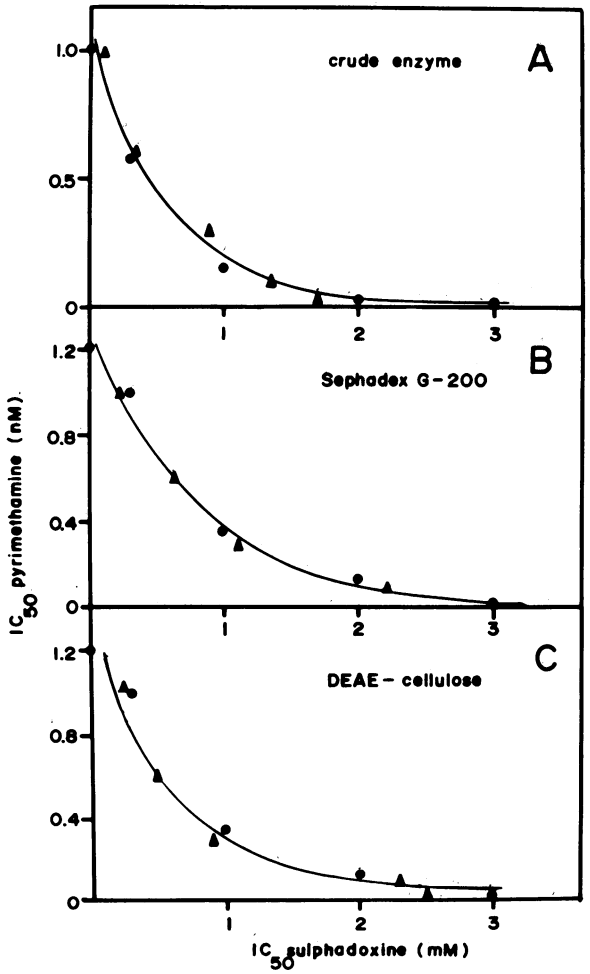

FIG. 4. Potentiating effect of pyrimethamine and sulfadoxine on *P. chabaudi* dihydrofolate reductase. The analysis described in the legend to Fig. 2 was used, except that enzyme of different purity was used. (A) Crude enzyme of pyrimethamine-sensitive *P. chabaudi*; (B) enzyme from the Sephadex G-200 column; (C) enzyme from the DEAE-cellulose column.

difficult to correlate the potentiation effect at the biological level with that at the enzyme level, the observation that sulfadoxine inhibited plasmodial dihydrofolate reductase and that there was a potentiation effect with pyrimethamine on the inhibition of this enzyme raises the possibility that the potentiation observed at the enzyme level may contribute to the potentiation at the biological level. The relative importance of various mechanisms, including the simultaneous inhibition of dihydrofolate reductase by the two drugs and the sequential inhibition of dihydropteroate synthetase and dihydrofolate reductase, remains to be further investigated.

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