INHIBITORY MECHANISM OF SULFAPHENAZOLE ON TOLBUTAMIDE ELIMINATION FROM PLASMA IN RAT*

YI-JONG LIN,** SHOJI AWAZU*** AND MANABU HANANO

Faculty of Pharmaceutical Sciences, The University of Tokyo, Hongo, Bunkyo-ku, Tokyo, Japan

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The inhibitory mechanisms of sulfaphenazole (SP) on tolbutamide (TB) elimination from plasma in the rat were examined. Three mechanisms, 1) inhibition of distribution of TB in the metabolic organ, the liver, 2) inhibition of excretion of TB in the urine and 3) inhibition of oxidation of TB in the liver were proposed. Only the third mechanism, of which SP inhibits the microsomal oxidation of TB was evidenced. The reason why SP, which is not oxidized *in vivo*, inhibits the oxidation of TB was explained by the finding that SP binds to hepatic cytochrome P-450. SP, showing the type II of P-450-substrate difference spectrum, gives a much stronger binding tendency than other sulfa drugs, such as sulfadimethoxine, sulfisoxazole and sulfamethoxazole, which have been reported to have little effect on TB elimination. It is suggested that a compound, not oxidized *in vivo*, can possibly have an effect on oxidative drug metabolism, and that a spectrum study of P-450-substrate complex is a useful tool to predict such an effect, *i.e.*, drug-drug interaction *in vivo*.

Keywords—tolbutamide-sulfonamides interactions; P-450 difference spectrum; binding to cytochrome P-450; inhibitory mechanism of drug oxidative metabolism; drug interaction

It is well known in man that sulfaphenazole[SP; N¹-(1-phenylpyrazol-5-yl)sulfanilamide] potentiates the hypoglycaemic activity of tolbutamide [TB; 1-butyl-3-*p*-tolylsulfonylurea] by increasing its half-life in plasma.¹⁾ Concerning the metabolism of TB, Thomas and Ikeda²⁾ have reported that TB is oxidized to hydroxytolbutamide [HTB; 1-butyl-3-*p*-hydroxymethylphenylsulfonylurea] and subsequently to carboxytolbutamide [CTB; 1-butyl-3-*p*-carboxyphenylsulfonylurea], in rat as well as in man. And Tagg *et al.*³⁾ have reported that the oxidation of TB to HTB occurs in the liver microsomal fraction of rat. In order to elucidate the inhibitory effect of SP, Schulz and Schmidt⁴⁾

examined the effect of SP on the half-lives of TB, HTB and CTB in man, respectively. They have concluded that SP inhibits the first oxidation step of TB to HTB, based on the results that SP increased the half-life of TB but had no effect on either HTB or CTB. Kristensen⁵⁾ also reported that TB was oxidized in human liver microsomes and that this step was blocked by SP. This effect has been reported recently as a competitive inhibition in the rabbit by Rowland and Matin.⁶⁾ The latter workers have also shown that the effect of SP on TB elimination from plasma in man can be analyzed pharmacokinetically with simple Michaelis-Menten type equations.⁶⁾ Pond *et al.* suggested that SP acts

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** Present address: Colgate-Palmolive Co., Research and Development Department, 909 River Road Piscataway, NJ 08854, U.S.A.

*** Present address: Tokyo College of Pharmacy, 1432-1 Horinouchi, Hachioji, Tokyo 192-03, Japan, to whom inquiries should be addressed.

by a direct inhibition of the microsomal mixed function oxidase system, however, in the near absence of the data, one may wonder how such a notion became established.⁷⁾ Although these past *in vivo* and *in vitro* results seem to show a definite mechanism for the effect of SP inhibition on TB, it is not yet clear as to how SP which is metabolized to a glucuronide and an acetylated compound but not oxidized *in vivo*⁸⁻¹⁰⁾ inhibits the microsomal oxidation of TB. And it is not answered why other sulfa drugs, such as sulfadimethoxine [SDM; N¹-(2, 6-dimethoxy-4-pyrimidinyl)sulfanilamide], sulfisoxazole [SI; N¹-(3, 4-dimethyl-5-isoxazolyl)sulfanilamide] and sulfamethoxazole [SM; N¹-(5-methyl-3-isoxazolyl)sulfanilamide] which have been reported to have little effect on TB.¹¹⁾ In this study, inhibitory mechanisms of SP on TB elimination from plasma in rat were examined and preliminary data of drug-drug interaction between TB and other sulfonamides (SDM, SI and SM) were presented.

EXPERIMENTS AND METHODS

Drug Administration and Sampling—Male albino rats (Donryu), weighing 250–285 g, were used. Femoral artery, femoral vein and bladder were cannulated with polyethylene tubing under ether anesthesia. A bolus injection of 0.509 mmol/kg of SP^{*1} was given into the femoral vein and the SP was infused continuously^{*2} at a rate of 0.131 mmol/kg/hr during the experiment to maintain the plasma level of SP as constant as possible. After the bolus injection was given, 80 mg/kg of TB^{*3} was given as a bolus into the other femoral vein. The administered solution contained 500 mg SP and 250 mg Na₂CO₃ or 500 mg TB and 300 mg Na₂CO₃ in 10 ml H₂O, respectively. When SP was not given at the control experiment, the corresponding volume of sodium chloride in-

jection was given as a bolus or infused. Other sulfa drugs, SDM^{*4}, SI^{*5}, SM^{*6}, were given similarly to SP. Blood and urine samples were drawn through the cannula at appropriate time intervals. For a liver sample, after the rat was cut on the carotid artery and killed by bleeding, the liver was removed as rapidly as possible and homogenized in an equal volume of tris-HCl buffer (pH 7.4) cooled in ice-water.

In Vitro Binding to Liver—A liver was taken as the above, and was perfused with isotonic sodium chloride solution for 30 min. The liver was homogenized as above and centrifuged at 100000-g^{*7} 2 hr. Three ml of the supernatant was put into Visking tubing (18/32 seamless) and dialyzed for 48 hr at 4° against 10 ml of pH 7.4 buffer containing drugs. The binding ratio was calculated by correcting for the blank value resulted from binding to the Visking membrane.

Binding to Plasma Protein—An epoxy resin plate which has five compartments of 12 mm depth and 26 mm diameter was used for the dialysis study. Two plates were joined tightly, holding a Visking membrane between them. Two ml of rat plasma was put into one compartment and 2 ml of phosphate buffer (pH 7.4) containing an appropriate concentration of drug was put into the other compartment. The plates were incubated for 48 hr at 4°. The binding ratio was calculated by correcting for the blank value resulted from binding to the Visking membrane.

Hydroxylation of TB in Liver Microsomes—Microsomes was prepared according to Sato.¹²⁾ The reaction mixture was prepared to contain 2.88 mg of microsomal proteins, 0.6 μmol of drugs, 10 μmol of MgCl₂ and 1 μmol of NADPH^{*8} in 1.15 ml of 0.05 M tris-HCl buffer (pH 7.4). The reaction mixture was incubated at 37° for 30 min and the reaction was stopped by adding

*1 Sulfaphenazole; Merian, Dainippon Pharmaceutical Co., Ltd., Osaka, Japan.

*2 Natsume model 12H automatic micro-infusion pump.

*3 Tolbutamide; products of Nakataki Pharmaceutical Co., Ltd., Tokyo, or Yamanouchi Pharmaceutical Co., Ltd., Tokyo, Japan.

*4 Sulfadimethoxine; Abcid, Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan.

*5 Sulfisoxazole; Daiichi Pharmaceutical Co., Ltd., Tokyo, Japan.

*6 Sulfamethoxazole; Sinomin, Shionogi Co., Ltd., Osaka, Japan.

*7 Hitachi 55P ultracentrifuge, 42000 rpm with 65P rotar.

*8 Nicotinamide-adenine-dinucleotide phosphate, reduced form; Boehringer Mannheim, catalogue number 15502.

0.3 ml of 3 N HCl. The amount of HTB produced was determined. The protein of microsome was determined with Lowry's method.¹³⁾

Elimination of NADPH from Microsome Reaction Mixture—Modifying the mixture of Statland *et al.*,¹⁴⁾ the reaction mixture was prepared as follows. To 5 ml of 0.05 M tris-HCl buffer (pH 7.4) either containing 6 μ mol sulfa drug or blank, were added 0.5 ml of the microsomal suspension (2.8 mg protein/ml of tris-HCl buffer, pH 7.4), 0.1 ml of $MgCl_2$ (100 μ mol/ml H_2O), and 0.03 ml of NADPH solution (20 μ mol/ml H_2O). The mixture was incubated at 37°, and the residual NADPH was determined as the difference in absorbance* between 340 and 380 nm.¹⁵⁾

Substrate Induced Difference Spectrum—The spectrum was observed* in the presence of 7.8 mg microsomal protein in 3 ml of tris-HCl buffer (pH 7.4) containing substrates against the buffer solution containing the same concentration of microsomes only.

Determination of TB and Its Metabolites—The separation of these compounds was carried out according to Shibasaki *et al.*,¹⁶⁾ and the colorimetric method of Spingler¹⁷⁾ was used for the determination, instead of the ultraviolet (UV) method of Shibasaki *et al.*, to avoid the UV absorption due to sulfa drugs.

TB in Plasma: One tenth ml of plasma was added to 0.6 ml of 0.5 M phosphate buffer (pH 5.0), and the mixture was shaken for 25 min with 5 ml of *n*-heptane/ $CHCl_3$ (4:1). After centrifugation, the organic layer was used for the TB determination. Four ml of the organic layer was evaporated under vacuum, then 0.8 ml of isoamylacetate and 0.1 ml of 0.1% dinitrofluorobenzene (DNFB) in isoamylacetate reagent were added to the residue, and the mixture was heated at 145° for 7 min. TB was determined at 395nm** as the compound which was produced from DNFB and *n*-butylamine (procedure 1).

Negative interference by HTB and CTB was verified by using pure HTB and CTB.

HTB in Plasma: Five tenth ml of the above aqueous layer was extracted with 5 ml of ethyl ether for 15 min and centrifuged for 15 min. Four ml of the ether layer was evaporated. To the residue was added 0.8 ml of isoamylacetate and 0.1 ml of DNFB reagent, and the absorbance of this fraction was determined similarly for TB as above (procedure 2). Since the rate of extraction of TB with *n*-heptane/ $CHCl_3$ in procedure 1 was 85%, the net absorbance of HTB was corrected for unextracted TB as follows: Net absorbance of HTB = observed absorbance in procedure 2 - (absorbance in procedure 1) $\times$ 0.128***. The contamination with CTB was not negligible, however, the plasma concentration of the metabolite was so small that no further separation procedure was carried out.

TB in Urine: Urine sample was diluted five times with water. To 0.5 ml of the diluted specimen were added 0.3 ml of pH 5.0 phosphate buffer and 5 ml of *n*-heptane/ $CHCl_3$ (4:1), and then TB was extracted and determined in a manner similar to that described for plasma samples.

HTB and CTB in Urine: Five tenth ml of the diluted urine sample were extracted with 0.3 ml of 0.5 M phosphate buffer (pH 6.0) and 4 ml of isoamylacetate. After centrifugation, to 3 ml of the organic layer was added 0.3 ml of 0.1% DNFB reagent, and the total amount of TB and HTB was determined. Subtracting the amount of TB from the total amount of TB and HTB allowed us to calculate the amount of HTB in the urine. After the extraction of 0.5 ml of the diluted urine sample with 4 ml of isoamylacetate in the presence of 0.3 ml of 3 N HCl, the total amount of TB, HTB and CTB was determined as above. From the results, the amount of CTB in the urine was calculated.

TB in Liver: The liver was homogenized in an equal volume of tris-HCl buffer (pH 7.4). One g of

* Hitachi two-wavelength double beam spectrophotometer, model 356.

** Micro cell of 1 $\times$ 35 $\times$ 10 (mm) was used.

*** For example, the absorbance of 10 μ g TB/0.1 ml plasma in procedure 1 was 0.125. Due to incomplete extraction, the TB which remained in aqueous layer produced an absorbance of 0.016 in procedure 2. The ratio of 0.128 (0.016/0.125) was needed to correct for the unextracted TB.

the homogenate was taken, 1 ml of phosphate buffer (pH 5.0) was added, and then extracted with 6 ml of *n*-heptane/ CHCl_3 (4:1). After centrifugation, to 5.5 ml of the organic layer was added 4 ml of 0.1 N NaOH. After shaking and centrifugation, to 3 ml of the NaOH layer were added 0.3 ml of 3 N HCl and 4 ml of isoamylacetate. TB extracted into isoamylacetate was determined as above for urine.

TB in 100000-g Supernatant of Liver: One tenth ml of the supernatant of the liver was taken, and TB was determined as above for plasma.

HTB in Microsomal Reaction Mixture: After the reaction was stopped with 0.3 ml of 3 N HCl, the mixture was extracted with 8 ml of CHCl_3 . Dividing the organic layer into 3 and 4 ml portions, each of these two fractions was evaporated under vacuum. Dissolving the residue of the 3 ml portion in 3 ml isoamylacetate, the total amount of TB and HTB was determined as above for urine. From the residue of the other portion, the amount of TB was determined in a manner similar to that described for plasma. From these results, the amount of HTB was calculated.

Determination of Sulfa Drugs—All sulfa drugs in the plasma and liver were determined according to the method of Tsuda.¹⁸⁾ No interference with TB and its metabolites was observed in the determination of sulfa drugs, and *vice versa*.

MATERIALS AND CHEMICALS

HTB—TB was administered by *i.p.* (250 mg $\times$ 4 doses) to five rats and the urine was collected. Ethereal extracts of urine were obtained according to Thomas and Ikeda.²⁾ The extract was evaporated and the residue was dissolved in 600 ml of 0.3 M Na_2CO_3 , which was placed on a Dowex 1-8X column. The eluate of 0.1 N HCl/EtOH (1:1) from the column was evaporated under vacuum. The residue dissolved in CHCl_3 was placed on a silica gel column, and eluted with CHCl_3 /MeOH (95:5). The eluate was evaporated under vacuum and the residue recrystallized from MeOH- H_2O . mp: 99–101° [98.5–100°³⁾; 101–103°¹⁹⁾; 110–110.5°²²⁾; 115–118°¹⁶⁾]. *Anal.* Calcd. for $\text{C}_{12}\text{H}_{18}\text{N}_2\text{O}_4\text{S}$: C, 50.40; H, 6.34; N, 9.77.

Found: C, 50.45; H, 6.41; N, 9.83.

CTB—According to Louis *et al.*,²⁰⁾ CTB was obtained from the urine of two volunteers who took 0.5 g of TB. mp: 216–218° [215–217°²⁰⁾]. *Anal.* Calcd. for $\text{C}_{12}\text{H}_{16}\text{N}_2\text{O}_5\text{S}$: C, 47.99; H, 5.37. Found: C, 47.87; H, 5.28. All solvents and reagent chemicals were purchased from commercial sources. Unless otherwise specified, all chemicals were reagent grade and used directly.

RESULTS AND DISCUSSION

Effect of SP on TB Elimination from Plasma

As shown in Fig. 1, SP which was maintained at about 400–500 $\mu\text{g}/\text{ml}$ in the plasma during the entire experimental period, markedly decreased the rate of elimination of TB from the plasma in the rat. The plasma half-life of TB increased from 3.1 hr of control rats to 8.0 hr of SP treated rats. A two compartment model was selected,²¹⁾ and the data were fitted to this model, using a nonlinear curve fitting program.²¹⁾ The computer estimated pharmacokinetic parameters are listed in Table I. Significant differences between control and SP treated rats were observed only for the elimination rate constant, k_{el} , ($p < 0.01$), while other constants did not differ significantly at the 5% level. It was clearly demonstrated that SP inhibits TB elimination from plasma. The observed concentrations of HTB in the control rats were so low that the data were not analyzed kinetically, however, such low concentrations could be in a good agreement with the results of Schulz⁴⁾ and Shibasaki *et al.*²²⁾ where the elimination of HTB was reported to be at least ten times more rapid than TB. On the SP treated rats the HTB level was too low to be detected by this assay method. From these results, it was suggested that the metabolism of TB to HTB could be depressed. Thus, less HTB appeared in the plasma of SP treated rats. Since it became clear that SP decreased the elimination of TB, the following three mechanisms were considered for the inhibiting effect of SP: 1) inhibition of distribution of TB in the metabolic organ, the liver, 2) inhibition of excretion of TB in the urine, 3) inhibition of oxidation of TB in the liver. These mechanisms were examined in the above order.

FIG. 1. Plasma Concentrations of Tolbutamide, Hydroxytolbutamide and Sulfaphenazole as a Function of Time in the Rat

(A): tolbutamide alone was administered intravenously. Each value is the mean $\pm$ standard error of 5 experiments. (B): tolbutamide was administered intravenously, while sulfaphenazole was simultaneously administered as described in the text. Each value is the mean $\pm$ standard error of 3 experiments. $\circ$, observed concentration of tolbutamide; $\square$, observed concentration of hydroxytolbutamide; $\bullet$, observed concentration of sulfaphenazole; $\bar{\square}$, standard error. Lines were calculated with parameters in Table I.

Effect of SP on TB Distribution to Liver

Table II shows the binding of TB to 100000-g liver supernatant at pH 7.4 (1 g wet weight/ml of pH 7.4 buffer). The unbound fractions of TB were 0.49 and 0.82 for the control, and 0.48 and 0.81 for the presence of SP at the free TB concentrations of 6.2 and 37 μg/ml, respectively. The unbound fractions of TB showed no significant difference between the control and those containing SP. These results suggested that the presence of 750 μg/ml of SP had no effect on the binding of TB to

100000-g liver supernatant which furnished the enzymes necessary to reduce NADP⁺ to NADPH in the oxidative metabolism. Accordingly, it could be anticipated that SP does not affect TB distribution in the liver. This conclusion was supported by *in vivo* studies. Fig. 2 shows the plasma concentrations and liver levels (μg/g wet weight of liver) of TB in the absence and the presence of SP, and of SP itself. By extrapolating the level of TB in the liver to zero time, the results were 155 μg/g and 153 μg/g in control rats and SP treated rats,

TABLE I. *Least Squares Estimates of Parameters^{a)} of Two-Compartment Model and Their Standard Errors obtained in the Nonlinear Least Squares Curve Fitting of Plasma Tolbutamide Concentration in the Rat*

	k_{12}	k_{21}	k_{el}	V_1	V_2
Control ^{b)}	3.80 ± 1.2	6.84 ± 1.7	0.363 ± 0.02	204 ± 12	113 ± 46
With ^{c)} sulfaphenazole	1.49 ± 0.4	3.40 ± 0.9	0.125 ± 0.01	225 ± 6	99 ± 38

a) Model is given in the chart below. k ; hr^{-1} , V ; ml/kg .
The computer estimated parameters $\pm$ standard errors.
Chart for Two-Compartment Model

b) Tolbutamide was given alone as a bolus intravenously (5 experiments).

c) Sulfaphenazole was infused continuously (3 experiments).

respectively. This suggested that TB was taken up by the liver to the same degree with or without the presence of SP. From these *in vitro* and *in vivo* results, SP showed no evidence of inhibitory activity on the distribution of TB in the liver. Thus, the first proposed mechanism by which SP inhibits TB distribution in the liver was ruled out.

Effect of SP on TB Excretion in Urine

The urinary excretion of TB and its metabolites in 48 hr are listed in Table III. HTB was the predominant metabolite excreted. About one third of the HTB in the control rats, and about half in the SP treated rats was excreted as CTB. Thomas²⁾ has reported that the main metabolite in the rat is HTB, and that CTB excretion is only a trace. By contrast, Wittenhagen *et al.*²³⁾ have shown that the amount of CTB excreted is larger than that of HTB. The results in this study seemed to lie between those of the two reports. In this study, less than 2% of TB was excreted both in the control rats and SP treated rats. Wittenhagen *et al.*²³⁾ stated that only a trace of TB was excreted. Since the amount excreted was so little, the direct process from plasma to urine was apparently not the main route of TB elimination from plasma. Therefore, the second proposed mechanism by which SP inhibits TB excretion in urine was also ruled out. The remaining mechanism is that by which the SP

effect is ascribed to the metabolic process, the third proposed mechanism.

Effect of SP on TB Hydroxylation in Hepatic Microsomes

The amounts of HTB produced from TB in microsomes are shown in Table IV. SP clearly inhibited TB hydroxylation, and this is in

TABLE II. *Effect of Sulfaphenazole on Tolbutamide Binding to 100000-g Supernatant of Liver Homogenates^{a)}*

	Control ^{b)}	With sulfaphenazole ^{c)}
$\frac{C_{free}^{d)}}{C_{total}}$	0.49	0.48
$\frac{C_{free}^{e)}}{C_{total}}$	0.82	0.81

a) Tolbutamide in 10 ml outer solution was dialyzed against 3 ml supernatant in visking tubing, in the absence or presence of sulfaphenazole. Each value is the mean of 2 experiments.

b) No sulfaphenazole was added.

c) Initial concentration of sulfaphenazole in outer solution was $750 \mu\text{g/ml}$.

d) Free tolbutamide concentration were $6.2 \mu\text{g/ml}$.

e) Free tolbutamide concentration were $37 \mu\text{g/ml}$.

FIG. 2. Liver Levels of Tolbutamide and Sulfaphenazole as a Function of Time

Each value is the mean $\pm$ standard error of 3 experiments.

○, tolbutamide liver level of control rats; △, tolbutamide liver level of sulfaphenazole treated rats; ●, sulfaphenazole liver level administered as the above; I, standard error. For comparison with plasma concentration, (a), tolbutamide plasma concentration of control rats; (b), tolbutamide plasma concentration of sulfaphenazole treated rats; (c), sulfaphenazole plasma concentration.

agreement with the findings of Kristensen⁵⁾ in human liver slices and of Rowland *et al.*⁶⁾ in rabbits. This inhibitory activity of SP seemed to support the third mechanism. However, it is not clear as to how TB oxidation is inhibited by SP which is not oxidized in liver.

For the microsomal oxidation of drugs, it has been well established that after the cytochrome P-450-substrate complex is reduced by an electron from NADPH, it is bound by oxygen, and then, the substrate is oxidized and P-450 is recovered.²⁴⁾ According to this scheme, two mechanisms can be considered: One is the effect of SP on NADPH oxidation in microsomes, and the other is the effect on the binding between P-450 and the substrate, and/or the reactivity of the P-450-substrate complex.

Effect of SP on NADPH Oxidation

As shown in Fig. 3, SP depressed the NADPH

oxidation. The percentages of residual NADPH were 72 and 61 with and without the presence of SP in 40 min. As mentioned previously, Dubach *et al.*¹¹⁾ have reported that other sulfa drugs, such as SDM, SI and SM have little effect on TB half-life in man. The effect of these sulfa drugs on the NADPH oxidation were also examined. The extent of these effects were similar to those of SP. Although SP depressed the NADPH oxidation slightly, the effect was considered too small to explain such a great inhibitory activity on TB oxidation of SP *in vitro* and *in vivo*, and the depression of NADPH oxidation which is also caused by several other sulfa

TABLE III. Urinary Excretion of Tolbutamide and Its Metabolites in 48 hr After 80 mg/kg of Tolbutamide was Administered intravenously in the Rat^{a)}

	Control ^{b)}	With sulfaphenazole ^{c)}
Tolbutamide	1 $\pm$ 0.1	2 $\pm$ 0.2
Hydroxytolbutamide	52 $\pm$ 4	38 $\pm$ 3
Carboxy-tolbutamide	18 $\pm$ 2	20 $\pm$ 2

a) Each value is the mean percentage of the dose $\pm$ standard errors.

b) Tolbutamide alone was administered (5 experiments).

c) Sulfaphenazole was given as a bolus and followed by intravenous infusion for 10 hr after tolbutamide was administered (3 experiments).

TABLE IV. Effect of Sulfaphenazole on Tolbutamide Hydroxylation in Rat Liver Microsomes^{a)}

Control	0.022 $\pm$ 0.005 μ mol
With sulfaphenazole ^{b)}	0.002 $\pm$ 0.002 μ mol

a) Amount of hydroxytolbutamide produced from 0.6 μ mol of tolbutamide in 1.15 ml of microsomal reaction mixture. Each value is the mean $\pm$ standard error of 9 determinations.

b) 0.6 μ mol of sulfaphenazole was added.

FIG. 3. *Effect of Sulfa Drugs on the Elimination of NADPH in Rat Liver Microsome Suspension*
Percentage of residual NADPH was plotted against time. Lines were drawn with bare eyes. ●, control; ○, with sulfaphenazole; ▲, with sulfadimethoxine; □, with sulfisoxazole; with sulfamethoxazole. Sulfa drug concentration was 1.07 mM.

drugs as shown in Fig. 3 cannot be described as the mechanism of the rather specific inhibition effect of SP on TB elimination from plasma.

Substrate-Induced Difference Spectra of P-450 caused by TB and Sulfa Drugs

It is well known²⁶⁾ that a compound induces a difference spectrum when it binds to P-450,

irrespective of whether it is oxidized or not, and the spectrum is classified as type I, type II and modified type II. If SP affects microsomal oxidation, it could be expected that SP binds to P-450 and induces a difference spectrum. The measurements of the difference spectra were carried out for TB and SP. As shown in Fig. 4, TB gave type I spectra and SP gave type II spectra. For comparison, the induced spectra of SDM, SI and SM were measured. They also showed type II spectra.

The dissociation constants (K_s) for the drug: P-450 complexes given in the equation $\Delta_{abs} = A[S]/(K_s + [S])$ were computed by a nonlinear least squares method from the data of spectral change. An example for SP and SM, the calculated and observed data for absorbance *vs.* concentration are shown in Fig. 5. As shown in Table V, the K_s values were 0.67, 1.22, 2.70 and 6.30 mM for SP, SDM, SI and SM respectively. Since SP gives the smallest K_s value among the four sulfa drugs, it suggests the strong binding tendency of SP which may explain the reason why SP inhibits the hydroxylation of TB *in vitro* and *in vivo*.

Effect of SDM, SI and SM on TB Elimination from Plasma

The preliminary studies of the effect of these sulfa drugs on TB elimination from plasma of the

FIG. 4. *Spectral Changes of Rat Liver P-450 caused by Tolbutamide and Sulfaphenazole were shown as Difference Spectra against the Control Microsome Suspension*
Curves, a, b and c correspond to different substrate concentrations shown in this figure.

FIG. 5. Difference Absorbance of P-450 induced by Sulfaphenazole and Sulfamethoxazole are shown

○, □ : sulfaphenazole; ●, ■ : sulfamethoxazole. Circles are for the first run and squares are for the second run. Data of two runs were analyzed with the equation of $\Delta_{abs} = A[S]/(K_s + [S])$, and lines are the calculated results.

TABLE V. Spectral Dissociation Constants (K_s) and Maximum Spectral Change (A) of Tolbutamide and Sulfa Drugs^{a)}

Substrate	K_s (mM)	A
Tolbutamide	6.74 ± 1.25	0.0221 ± 0.0021
Sulfaphenazole	0.67 ± 0.05	0.0207 ± 0.0005
Sulfadimethoxine	1.22 ± 0.26	0.0204 ± 0.0010
Sulfisoxazole	2.70 ± 0.91	0.0205 ± 0.0022
Sulfamethoxazole	6.30 ± 0.85	0.0139 ± 0.0009

a) Spectral data were analyzed with the equation of $\Delta_{abs} = A[S]/(K_s + [S])$, where Δ_{abs} is observed data, $[S]$ is substrate concentration. Each value is the estimated parameters $\pm$ standard error of 2 experiments.

rat were examined. After the sulfa drugs and TB were administered the time course for TB plasma concentration was obtained and analyzed similarly as in SP treated experiments. These results are shown in Fig. 6 and Table VI.

In Table VI, it can be seen that SDM decreased

FIG. 6. Plasma Concentrations of Tolbutamide as a Function of Time in the Rat

A: sulfadimethoxine treated rats, each value is the mean $\pm$ standard error of 3 experiments, B: sulfisoxazole treated rats, each value is the mean $\pm$ standard error of 2 experiments, C: sulfamethoxazole treated rats, each value is the mean $\pm$ standard error of 2 experiments. For other caption, see Fig. 1.

TB elimination, however, SI and SM increased TB elimination. As shown in Table V, SDM had the second smallest K_s value among the four sulfa drugs. The smaller inhibitory activity of SDM compared to that of SP on TB elimination supported the conclusion that the effect of sulfa drugs on the TB elimination relate] to their binding tendency to P-450. The reason why Dubach *et al.*¹¹⁾ did not find out such an effect could be due to differences in species and method of administration. Although the promoting effect of SP on the TB elimination has been reported in the rabbit,²⁷⁾ the promoting effect of SI and SM on the TB elimination could be explained by the displacement of the protein-bound TB in plasma. The binding of the sulfa drugs to the undiluted rat plasma was measured and the results are shown in Fig. 7 and Table VII. The K_p values were 11.8, 15.7, 19.4 and 4.31 l/mmol for SP, SDM, SI and SM respectively. It can be seen that SI exhibits stronger binding to plasma protein than those of SP and SDM. However, SM showed little binding tendency to plasma protein. More experiments would be necessary to clarify these TB-SI and TB-SM drug-drug interactions. From these results, it is suggested that sulfa drug which has a strong binding tendency to microsome P-450 can have an inhibitory effect on the TB elimination from plasma.

The question arises as to how SP, the type II compound inhibits the oxidation of TB, the type I

compound. In fact, such findings have been reported. Schenkman *et al.*²⁸⁾ have reported that nicotinamide (type II compound) inhibits aminopyrine (type I compound) demethylation with P-450, and since then, many researchers have omitted nicotinamide which had been routinely added to microsomal reaction mixtures. As the competitive inhibition of SP has been reported for microsomal oxidation in the rabbit,⁶⁾ displacement of TB from the binding site of microsomes by SP is considered as an acceptable mechanism. Such a displacement could be expected from the results of Kitagawa *et al.*²⁹⁾ that nicotinamide can bind to type I site and exhibit type I spectrum at very low concentrations (0.05 mM). If SP can bind to type I site at low concentration as nicotinamide, it would displace TB from there easily. Displacement with such a binding could also be expected from the results of Kamataki *et al.*³⁰⁾ that most of type I compounds can bind to both type I and II binding sites. SP effect could be explained, if TB binds to both type I and II binding sites and a fraction of those bindings is displaced by SP. On the other hand, Netter *et al.*³¹⁾ have suspected an allosteric interaction between two sites and a competition of binding to type II site with oxygen to explain why metyrapone (type II compound) inhibits the microsomal hydroxylation of type I compound such as aminopyrine. Sasame and Gillette³²⁾ have suggested the slowing down of the reduction of the cytochrome P-450-substrate

TABLE VI. *Least Squares Estimates of Parameters^{a)} of Two-Compartment Model and Their Standard Errors obtained in the Nonlinear Least Squares Curve Fitting of Plasma Tolbutamide Concentration in the Control Rats and Sulfonamides Treated Rats*

	k_{12}	k_{21}	k_{el}	95% confidence limits of k_{el}	V_1	V_2
Control	3.80 ± 1.2	6.84 ± 1.7	0.363 ± 0.02	0.415—0.311	204 ± 12	113 ± 46
SDM ^{b)}	0.74 ± 0.2	1.81 ± 0.3	0.225 ± 0.01	0.242—0.208	198 ± 6	81 ± 23
SI ^{c)}	2.86 ± 2.7	6.90 ± 4.7	0.536 ± 0.04	0.636—0.436	185 ± 14	76 ± 89
SM ^{d)}	4.75 ± 1.4	11.8 ± 1.1	0.593 ± 0.04	0.696—0.490	163 ± 12	65 ± 20

a) For the legend other than below, see Table I.

b) Sulfadimethoxine treated rats (3 experiments).

c) Sulfisoxazole treated rats (2 experiments).

d) Sulfamethoxazole treated rats (2 experiments).

FIG. 7. Sulfa Drugs binding to Undiluted Rat Plasma

The abscissa shows the free drug concentration (C_f) and the ordinate shows the bound drug concentration (C_b). Lines were calculated with the equation of $C_b = n[P]K_p C_f / (1 + K_p C_f)$, using the parameters in Table VII, and circles are the observed data.

TABLE VII. The Binding Constants of Sulfa Drugs to the Undiluted Rat Plasma

	SP	SDM	SI	SM
$n[P]$ (mmol/l)	1.06	1.27	0.752	0.753
K_p (1/mmol)	11.8	15.7	19.4	4.31

The equation $C_b = n[P]K_p C_f / (1 + K_p C_f)$ was used. Where, C_b is the concentration of bound drug, C_f is the concentration of free drug, n is the number of binding sites, $[P]$ is the concentration of plasma protein and K_p is the binding constant to plasma protein.

complex²⁵⁾ and the alteration of the affinity of type I site for a substrate as the cause for type II compounds to inhibit the oxidation of type I compounds. These proposed mechanisms could explain why SP inhibits TB microsomal oxidation.

From the present findings that the strong binding tendency of SP to microsomal P-450 at type II

site can explain the inhibitory activity on oxidative TB elimination *in vivo*. It suggested that a compound which is not oxidized *in vivo* can possibly effect on oxidative drug metabolism, and the measurement of the P-450 spectrum is a useful tool to predict such an effect, *i.e.*, drug-drug interaction *in vivo*.

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