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Effect of 5-*n*-Butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine on the Sulfonamides Transfer in the Rat

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Effect of 5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (BCP), an anti-inflammatory agent, on the sulfonamide transfer in the rat has been investigated with special attention to the protein binding. Four sulfonamides used in the experiment were sulfamethoxazole, sulfaphenazole, sulfisoxazole, and sulfamethizole.

Protein binding and the extent of displacement of a sulfonamide by BCP were studied both *in vivo* and *in vitro* techniques using fresh rat plasma.

Effect of BCP on the sulfonamides transfer in the rat was investigated in terms of the change of the apparent biological half-life period by the analyses of urinary excretion rate and blood level data. Apparent biological half-life periods of sulfonamides, displaced by BCP to a lesser extent in the *in vitro* studies, were hardly changed at all, whereas those of sulfonamides, displaced highly by BCP in the same condition, were significantly decreased except sulfamethizole. For the latter compound, it is conceivable that rapid elimination by itself obscures the effect of displacement by BCP.

Direct action of BCP on the active renal tubular transport as well as the acetylation of sulfonamides could be ruled out by the apparent lack of the effect of BCP on the accumulation of sulfonamides to the rat kidney cortical slices and the rate of sulfonamide acetylation by acetone powders of the pigeon liver.

Displacement from protein binding of one drug by another is well known, particularly in the case of weakly acidic drugs.[2] In most instances, displacement has been demonstrated by *in vitro* experiment. McQueen demonstrated the phenomena of displacement by using *in vivo* analyses in rats.[3] Anton reported the effect of displacing agents on a wide variety of drugs *in vivo*.[4] There are also instances of biopharmaceutical drug interactions in which displacement seems likely to have played a significant part. Anti-inflammatory agents are used clinically to prevent the inflammatory responses induced by microbial infections. Some of the anti-inflammatory agents are highly bound to plasma protein[5] and this characteristics may modify the bio-transfer of the anti-microbial agents by interfering with their binding *in vivo*.

This investigation was undertaken to determine whether the transfer of sulfonamides in rat could be modified by the administration of 5-*n*-butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine, a non-steroid anti-inflammatory agent known to have a long biological half-life (13—14 hours)[6] and to be highly bound to serum albumin[7] and to possess a high displacement potential, with particular attention to the protein binding.

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1) Location: *Yoshida-shimoadachi-cho, Sakyo-ku, Kyoto.*
2) B.B. Brodie, *Proc. Roy. Soc. Med.*, **58**, 946 (1965); M. C. Mayer and D.E. Guttman, *J. Pharm. Sci.*, **57**, 895 (1968); H.M. Solomon, J.J. Schrogie, and D. Williams, *Biochem. Pharmacol.*, **17**, 143 (1968); C.M. Kunin, *Clin. Pharmacol. Therap.*, **7**, 166 (1966); C.M. Kunin, *J. Lab. Clin. Med.*, **65**, 416 (1965); P.M. Keen, *Brit. J. Pharmacol.*, **26**, 704 (1966).
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Experimental

Materials——5-*n*-Butyl-1-cyclohexyl-2,4,6-trioxoperhydropyrimidine (BCP): Takeda Chemical Industries, Ltd., Sulfamethoxazole (SM): Shionogi Pharmaceutical Company, Ltd., Sulfaphenazole (SP): Dainippon Pharmaceutical Company, Ltd., Sulfisoxazole (SI): Shionogi Pharmaceutical Company, Ltd., Sulfamethizole (SMZ): Eisai Company, Ltd. were used as received. Albumin Human Fraction V: Nutritional Biochemical Corporation, Visking Dialysis Tubing:Visking Company. Rat Plasma:Plasma was prepared from blood samples in our laboratories. Rat was anesthetized with ether and blood samples were collected from the carotid arteries with plastic tube in a sodium heparin container, centrifuged at 2000 g for 30 minutes, and the separated plasma was used for binding experiments for undiluted one. This plasma was diluted with 1/15M phosphate buffer, pH 7.4, to yield the diluted one. All other chemicals were of analytical grade.

Binding Experiments——*In vitro* equilibrium dialysis was performed in the test-tubes by suspending sacs made of Visking dialysis tubing, which contain the drugs to be tested and 2 ml of 3% human albumin or undiluted rat plasma or 1:6 diluted rat plasma solution in 4 ml of 1/15M phosphate buffer, pH 7.4. Dialysis was carried out for 4 days at 4°. At the end of dialysis, an aliquot of buffer solution outside the sac was pipetted to be tested. Concentration of drugs used here ranged from 2.5×10^{-5} to 5.0×10^{-4}M. Details have been described in an earlier publication.[7]

Acetylation of Sulfonamides——Fresh pigeon livers, extensively used in the *in vitro* analysis of the acetylation reaction, were homogenized in a Waring blender with cold acetone and filtered on a Büchner funnel. The residue was homogenized again in cold acetone, filtered as above, and dried in a cold room under reduced pressure. The acetone powders were dissolved to a final concentration of 60 mg/ml in 0.15M potassium fluoride. Insoluble materials were removed by centrifugation at 4000 rpm for 5 minutes. The supernatant brown fluid, *i.e.* liver extraction fluid, was used to tests. The composition of the medium used in the acetylation of sulfonamides was as follows; 1 ml of liver extraction fluid, 0.02M potassium citrate, 0.02M potassium acetate, 0.02M phosphate buffer, pH 7.4, 0.0025M adenosine triphosphate, and 0.001M sulfonamide (all were final concentrations). Five milliliter of the medium was incubated at 37° for one hour. After incubation, an aliquot of the medium was pipetted to determine the unchanged drug.

Drug Accumulation by Rat Kidney Slices——In general, the technique of Cross and Taggart[8] was followed except for the following modifications. Renal cortical slices of rat were prepared with a safety razor blade in a cold room at 4° and weighed on a torsion balance immediately (initial wet weight) and again at the end of incubation period (terminal wet weight). Approximately 100—150 mg of tissue was cut from the kidney. Slices were incubated with air in a gas phase at 25° for a given period of time in 3 ml of the medium, 2.7 ml of which contained 0.87 ml of 0.3M sodium chloride, 0.2 ml of 0.1M sodium phosphate buffer of pH 7.4, 0.36 ml of 0.3M potassium chloride, 0.1 ml of 0.02M calcium chloride, 0.2 ml of 0.0001M sulfonamide, and water to a final volume of 2.7 ml. The concentration gradient of sulfonamide existing at the end of incubation (S/M ratio for sulfonamide) was calculated as the ratio of the concentration of free sulfonamide per milliliter of medium. To test the effect of BCP on the accumulation of sulfonamide by kidney slices, sulfonamide and the equivalent amount of BCP were incubated with slices as above. For the inhibitor of sulfonamide accumulation, 0.3 ml of 0.45 mM 2,4-dinitrophenol was used. Control observations in rats were examined by using *p*-aminohippurate.

Urinary Excretion——Sixty mg/kg of SM (or SP) was administered orally to rat with sonde. Exactly 4.5 hours after administration of SM (or SP), 5 times this dose of BCP was given orally as above. In the case of 60 mg/kg of SI (or 20 mg/kg of SMZ), 10 times this dose of BCP was added orally, exactly 1.5 hours (or 1.0 hour) after administration of SI (or SMZ). Urine samples were collected in a beaker with inhalation of ether[9] at 1.0 or 1.5 hour intervals for 24 hours, and urine pH was measured immediately. Suitably diluted urine samples were assayed for free sulfonamides colorimetrically. During experiments, rat was kept in the modified Bollman's cage.[10] After collection of urine samples, 2 ml of saline solution was given peritoneally.

Blood Levels——Sulfonamide was injected into the femoral vein in the rat and BCP was given intravenously prior to, or with, or posterior to, the administration of sulfonamide. Blood samples were collected into a small beaker by cutting off the end of the tail of rat at frequent intervals.[11] An aliquot of blood samples was hemolyzed in water, removed from protein with trichloroacetate. After centrifugation, supernatant fluid was separated to assy for free sulfonamide.

Analytical Methods——Analytical determinations of four sulfonamides were carried out by the colorimetric method as follows. Samples were acidified with 1N HCl, diazotized in the following regular manner, coupled with 2-diethylaminoethyl-1-naphthylamine and if necessary, extracted with isoamyl alcohol and

8) R.J. Cross and J.V. Taggart, *Am. J. Physiol.*, **161**, 181 (1950).
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their optical densities were determined, using Shimadzu spectrophotometer type QR-50. Absorption maxima in mμ were SM, 550; SP, 538; SI, 545, and SMZ, 550. For the urine or tissue samples, the same analytical methods were carried out for each diluted one. For the blood samples, the supernatant fluid described in the paragraph of 'Blood Levels' was assayed for free sulfonamides. For the slice samples, the supernatant fluid obtained as follows was used for assay. Slices were homogenized with 3 ml of 10% trichloroacetic acid and 2 ml of water. The slightly turbid suspending medium was treated in a similar manner. The solutions were centrifuged and the supernatant separated was assayed for free sulfonamides. A respective blank solution treated in the identical way served as a control.

Result and Discussion

Binding of Sulfonamides

Binding of four sulfonamides at pH 7.4 was determined by the equilibrium dialysis in 1/15M phosphate buffer solution using human albumin and rat plasma. The results are shown in Fig. 1, which indicates the relationship between free drug concentration and percentage bound of sulfonamides. Assuming the concentration of albumin[12] in rat plasma as about 3%,[13] comparison was made with SM binding between 3% human albumin and undiluted (100%) rat plasma. As is evident from the figure, essentially the same pattern was observed. Since diluted rat plasma (1:6) gave the results similar to the one obtained by undiluted rat plasma, the former was used throughout this experiment. The binding of other three sulfonamides by the 1:6 diluted rat plasma was also shown in Fig. 1. It is known that the binding tendency varies significantly from drug to drug and to the animal species,[14] and that protein binding of sulfonamides is affected by the dilution,[15] and further, that the use of ether as an anesthetic at the time of blood collection lowers the extent of binding.[16] In these experiments, relative values under the same conditions are of necessity. Therefore, diluted rat plasma was used throughout this experiment.

Binding of BCP to 1:6 diluted rat plasma is also shown in the same figure.

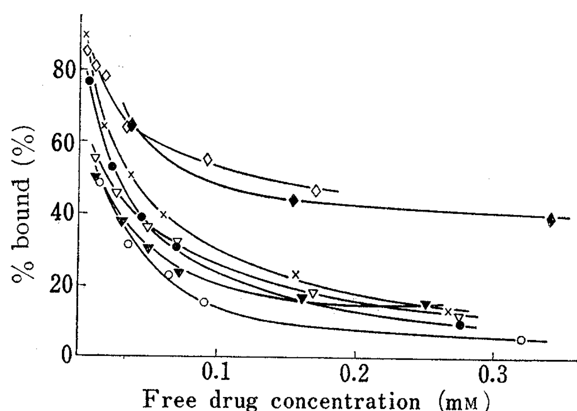

Fig. 1. Binding of Sulfonamides and BCP

binding of SM to 3% human albumin (◆) and to undiluted rat plasma (◇); binding of SM (○), SI (●), SP (▼), SMZ (▽), and BCP (×) to 1:6 diluted rat plasma

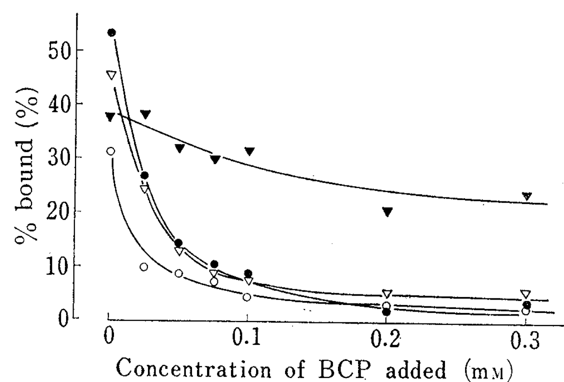

Fig. 2. Effect of BCP on the Binding of Sulfonamides to Rat Plasma

—○—: SM —●—: SI
—▽—: SMZ —▼—: SP

12) The binding of sulfonamides to plasma protein is known to be mainly to "albumin."
13) L.E. Scheving, J.E. Pauly, and T. Tsai, *Am. J. Physiol.*, **215**, 1096 (1968).
14) E. Genazzani and G. Pagnini, *Am. J. Vet. Res.*, **24**, 1212 (1963); J.L. Kucera and F.J. Bullock, *J. Pharm. Pharmacol.*, **21**, 293 (1969); J.A. Sturman and M.J.H. Smith, *J. Pharm. Sci.*, **57**, 895 (1967); D.T. Witak and M.W. Whitehouse, *Biochem. Pharmacol.*, **18**, 971 (1969).
15) A.H. Anton, *Clin. Pharmacol. Therap.*, **9**, 561 (1969).
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Displacement by BCP

Fig. 2 shows that the fraction of 0.05 mM sulfonamides bound by the 1:6 diluted rat plasma decreases with the increase of BCP concentration.

Percentage displacement of 0.05 mM sulfonamides by equi-molar concentration of BCP was 78.1% for SMZ, 72.9% for SI, 71.3% for SM, and 15.2% for SP. Expressed in terms of the amount displaced of sulfonamides, these values correspond to 0.018, 0.019, 0.012, and 0.003 mM respectively as shown in Fig. 3.

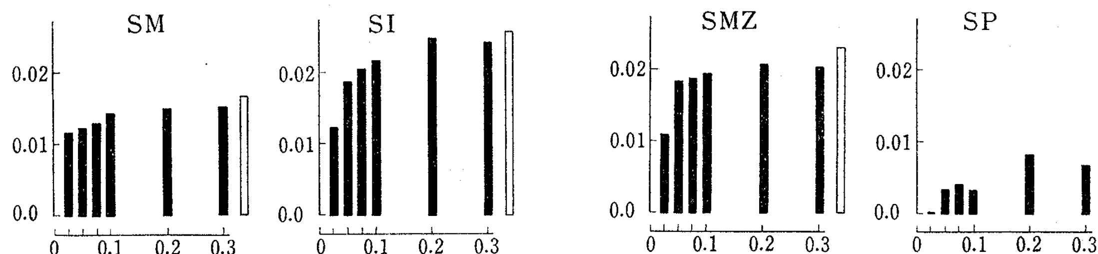

Fig. 3. Displacement of 0.05 mM Sulfonamides from Diluted Rat Plasma by BCP

Abscissa, concentration of BCP added (mM); ordinate, amount of sulfonamide displaced by BCP from diluted rat plasma(mM). □ in the histogram indicates the amount of each sulfonamide at 0.05 mM bound by the 1:6 diluted rat plasma.

This result shows that the percentage displaced from the binding sites is not always in proportion to the increase in the amount of drug unbound and the latter rather than the former is more important when considering the drug transfer in the body, although it is necessary to consider the binding constant, binding capacity, and other factors. However, judging from the protein binding data only, it is probable that blood levels of SP are hardly affected and, on the contrary, those of the others are significantly influenced by BCP.

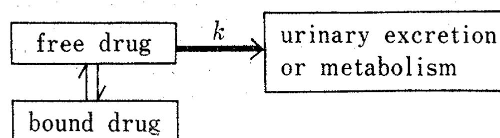

Chart 1

Process of drug transfer in the post-absorptive phase may be represented as given in Chart 1.

Here the following assumption is taken into consideration, (a) in the protein binding of drug, saturation is not brought about, (b) drug in the body is distributed into blood and the other extravascular compartment which spontaneously equilibrate with blood, (c) free and bound forms of drug are in rapid equilibrium in the body. The rate of elimination of unchanged drug is related to the concentration of free drug rather than that of the total drug in the blood. Fraction of free drug depends on the extent of protein binding. As the drug bound by protein can not pass across the membrane, protein binding both in blood and the other compartment influences the drug transfer in the body. The apparent elimination rate constant of drug "k_{app}" is given by equation (1),

$$k_{\text{app}} = k(\text{true elimination rate constant}) \times f \qquad (1)$$

where "f" means the fraction of free drug.

Therefore, in case where protein binding must be taken into consideration in the process of drug transfer, apparent elimination rate constant may be generally less than true one and hence apparent biological half-life (BHL) will be increased.

Sulfonamide to be tested was administered to rat orally and BCP was given orally after a given period of time. Effect of the latter on the apparent elimination rate of the former was investigated. Apparent BHL values of sulfonamides were calculated on the basis of urinary

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excretion rate analyses of the unchanged drug. Fig. 4 shows the results with SM. Urinary excretion rate *vs.* time curve for single 60 mg/kg administration of SM is given on the left hand side. Also shown is the effect of BCP administration on the apparent BHL value of SM on the right hand side of Fig. 4.

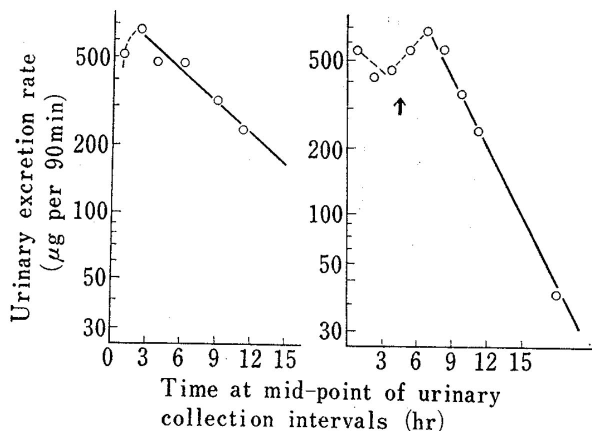

Fig. 4. Effect of BCP on the Elimination of SM in the Rat

Arrow in the right hand graph indicates the time when the BCP was given orally to rat. Peak appeared after administration of BCP shows that free SM in blood was increased by the displacement of SM bound to protein by BCP. The height of peak depended on the extent of displacement by BCP, BHL value of SM and others. Dose and the time of administration of BCP were determined by considering the BHL value of SM.

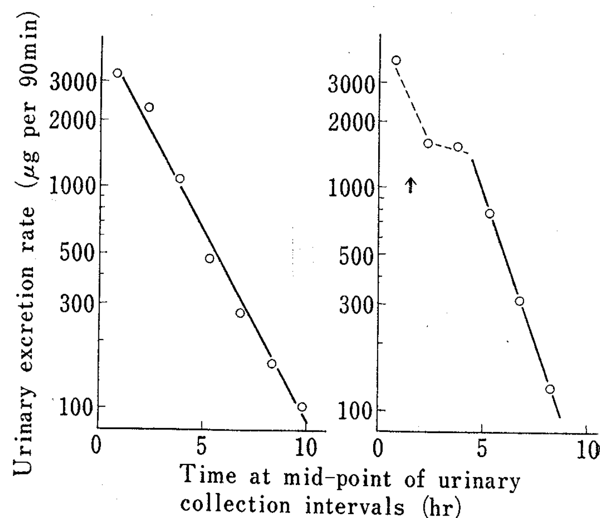

Fig. 5. Effect of BCP on the Elimination of SI in the Rat

Arrow in the right hand graph indicates the time when the BCP was given orally to rat. Dose and the time of administration of BCP were determined by considering the BHL value of SI.

Three hundred mg/kg dose of BCP was given orally 4.5 hours after the oral administration of 60 mg/kg of SM. Apparent BHL values of SM were 6.62±0.41 hr. and 2.92±0.41 hr respectively. Similar results were obtained in the case of SI. Rat was given 60 mg/kg dose of SI first and after 1.5 hours, ten times this dose of BCP was given orally. In this case, it was observed that the reduction of BHL value was about 41% of the control values. This result is shown in Fig. 5.

As was expected from the results of the *in vitro* binding experiments that the rat plasma binding constant for SP based on the data shown in Fig. 1 was larger than that for BCP significant effect of BCP was not observed in the case of SP. Administration of 300 mg/kg dose of BCP, five times that of SP, caused only 11% reduction in BHL value (Fig. 6).

Despite the strong displacement observed *in vitro*, effect of BCP on BHL value of SMZ was also not remarkable and only about 14% reduction was observed (Fig. 7).

These results are summarized in Table I.

It is apparent from the Table that the BHL values of sulfonamides which are displaced by BCP highly *in vitro* experiment were decreased significantly *in vivo* except SMZ. That the latter compound is excreted very rapidly *via* kidney in rat is well documented and this characteristics seems to obscure the *in vivo* displacement effect on the apparent elimination rate of SMZ. These changes in the apparent BHL values were also observed by the blood level analyses about SM as shown in Fig. 8.

Although protein binding displacement may be the cause of the change of apparent BHL values in rat, several other possibilities such as effect of BCP on the metabolism or urinary excretion of sulfonamides remained. BCP may alter the metabolic pattern of sulfonamides in the liver or renal excretion of unbound sulfonamides.

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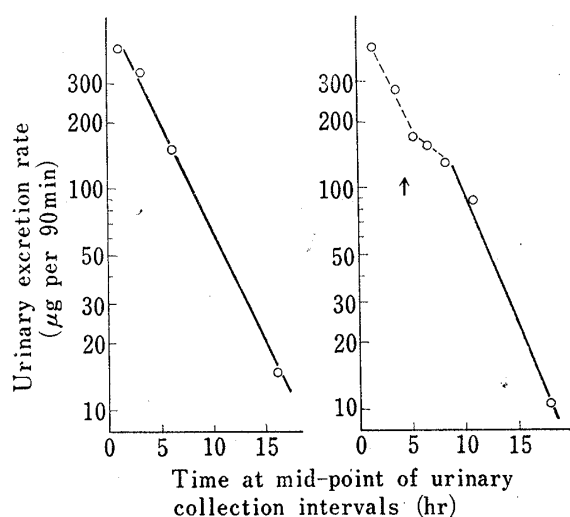

Fig. 6. Effect of BCP on the Elimination of SP in the Rat

Arrow in the right hand graph indicates the time when the BCP was given orally to rat. Dose and the time of administration of BCP were determined by considering the BHL value of SP.

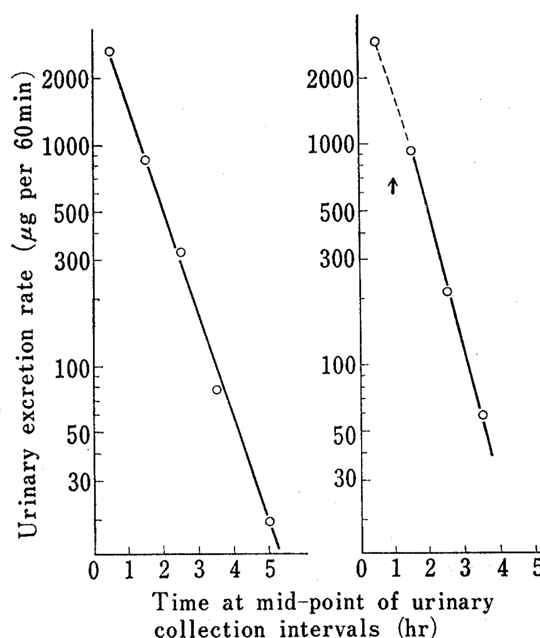

Fig. 7. Effect of BCP on the Elimination of SMZ in the Rat

Arrow in the right hand graph indicates the time when the BCP was given orally to rat. Dose and the time of administration of BCP were determined by considering the BHL value of SMZ.

Table I. BHL Values of Sulfonamides

	Biological half-life of sulfonamides (hr)		Dose (mg/kg)	
	Sulfonamides only	Sulfonamides with BCP	Sulfonamides	BCP
Sulfamethoxazole	6.62 ± 0.41	2.92 ± 0.41[a]	60	300
Sulfisoxazole	1.81 ± 0.27	1.06 ± 0.10[a]	60	600
Sulfamethizole	0.65 ± 0.06	0.56 ± 0.07[b]	20	200
Sulfaphenazole	3.17 ± 0.25	2.81 ± 0.21[c]	60	300

a) $p < 0.001$, *b)* $p < 0.02$, *c)* $p < 0.05$

With the above in mind, it became necessary to investigate the effect of BCP on the metabolism and the urinary excretion of sulfonamides. The former was examined by determing the acetylation of sulfonamides by acetone powders of pigeon liver. It was found that acetylation, the main biotransformation reaction in sulfonamides, was hardly affected at all by BCP.

Effect of BCP on the renal excretion of sulfonamides was investigated using kidney slice accumulation technique. If BCP affects directly on the kidney to accelerate the renal tubular secretion of sulfonamides, administration of BCP may bring about decrease in the apparent BHL values of sulfonamides. As is evident from Table II and Fig. 9, it was observed that BCP did not change the renal cortical slice uptake pattern of sulfonamides except SMZ. In the case of SMZ which is known to be actively secreted *via* kidney, BCP blocks slightly the accumulation of SMZ by rat kidney slices. And this means that the elimination of SMZ from kidney might be delayed, and hence that the BHL value of SMZ may increase. This, together with the rapid elimination of SMZ, may have compensated the rather strong protein

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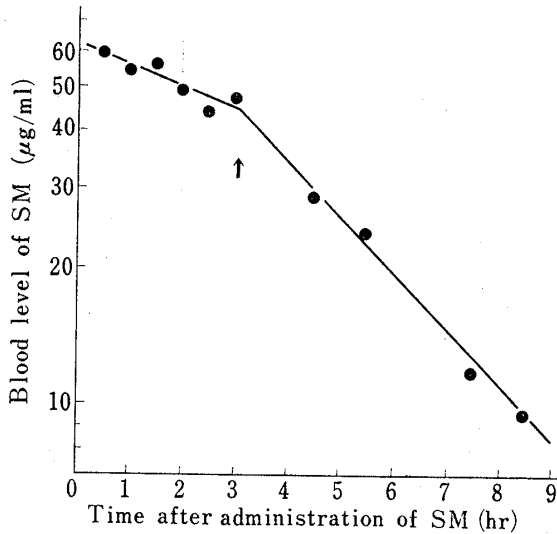

Fig. 8. Effect of BCP on the Blood Level of SM (*i.v.*) in the Rat

Arrow indicates the administration of BCP. By the effect of BCP, the BHL value of SM decreases from 6.2 to 2.5 hours. Similar results were obtained in such cases that BCP was given intravenously prior to, or with the administration of SM.

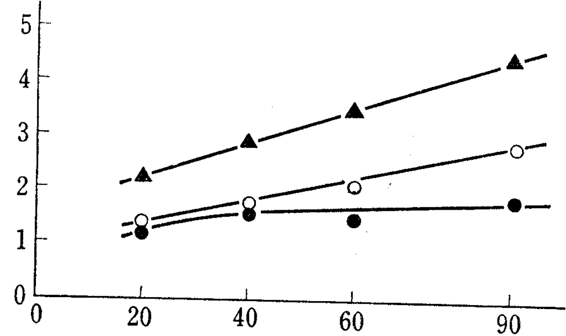

Fig. 9. The Accumulation of SMZ by Rat Kidney Slices

abscissa, time incubated (min); ordinate, S/M ratio
Each datum represents the mean of 3 to 9 observations. Time course of SMZ accumulation by rat kidney slices indicates that each curve dose not cross during the incubation for 90 min.

—▲—: SMZ only
—○—: SMZ+BCP
—●—: SMZ+DNP

Table II. Accumulation of Sulfonamides by Rat Kidney Slices (S/M Ratio)

Drug added	SMZ	SM	SP	SI	PAH
None	3.49	1.57	2.30	1.62	7.30
DNP	1.56	1.75	2.65	1.53	1.94
BCP	2.15	1.79	2.75	1.53	—

Each datum represents the mean of 3 to 9 observations. PAH (*p*-aminohippurate) was utilized as a control in this experiment. DNP (2,4-dinitrophenol) was used as an inhibitor of drugs accumulated actively on kidney slices. This result shows that drugs except SMZ are not affected by DNP and BCP.

binding displacement of SMZ by BCP. These kidney slice accumulation data may be rationalized on the basis that the values of S/M in the control experiment using *p*-aminohippurate as a maker substance agreed with the one obtained by Cross and Taggart.[8)]

Above results demonstrate the importance of the need to consider protein binding as a necessary parameter in the characterization of drug behavior.

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