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Studies on Drug Metabolism. XI.[1] Metabolic Interaction of Aniline and Phenylbutazone on the Liver Microsomal Drug Metabolizing Enzymes

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It has generally known that combinations of drugs often gave repressive effects on each metabolic rate.

Here, we report the metabolic interactions of aniline and phenylbutazone hydroxylations.

Simultaneous addition of aniline and phenylbutazone as the substrates to the same incubation mixture showed increased activity of phenylbutazone hydroxylation, on the other hand, activity of aniline hydroxylation was reduced than the activities obtained from the additions of each substrate respectively.

The increased phenylbutazone hydroxylation was found not only in the case of addition of *p*-aminophenol, one of the metabolites of aniline, to the same incubation mixture, but in the livers of rats received aniline intraperitoneally relatively short time before sacrifice.

When in need of drug administration, patients generally receive more than one drug simultaneously. Thus, it is important to study the metabolism of a drug in the presence of another, rather than investigating the two drugs separately.

Studies on the simultaneous effects of two compounds to drug enzyme system *in vitro* have been reported using an inhibitor and a drug.[3-5] These studies have shown only the inhibitory effects of so-called inhibitors on the metabolic rate of the drug, but the metabolic rate of inhibitors have been untouched.

Niwa, *et al.*[6] reported the effect of simultaneous administration of N-acetyl-*p*-aminophenol and phenylbutazone or its oxidized analogues on the biologic half life of the former, and estimated the exhibition of the interaction in the combination.

1) Part X: H. Kitagawa and H. Kadosawa, *Yakugaku Zasshi*, **89**, 1412 (1969).
2) Location: *1-33 Yayoi-cho, Chiba-shi, Chiba.*
3) K.J. Netter, "Proceeding of the First International Pharmacological Meeting, 6, Metabolic Factors Controlling Duration of Drug Action," edited by B.B. Brodie and E.G. Erdos, Pergamon Press, 1961, p. 214.
4) B.B. Brodie, *J. Pharm. Pharmacol.*, **8**, 1 (1956).
5) J.J. Burns, R.K. Rose, S. Goodwin, J. Reichenthal, E.C. Horning and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **113**, 481 (1955).
6) H. Niwa and T. Nakayama, *Yakugaku Zasshi*, **88**, 838 (1968).

This paper describes the metabolic interactions between two drugs or their metabolites when they were pipetted into the same vessel.

Ohshima, *et al.*[7] have already reported that there may be more than two different drug metabolizing enzymes such as aniline hydroxylase and hexobarbital hydroxylase in liver microsomes.

We wish to discuss the following data from a similar viewpoint.

Experimental

1. Animals——Male and female rats of Wistar strain weighing about 190 g were used. Livers from 10 rats were pooled and used for each enzyme assay. The results of revealed experiments were supported by 1—2 other repeated experiments. The data were expressed as relative values of control.

2. Route of Administration and Quantities of the Agents——All animals were injected intraperitoneally. The dose of each drug was 40 mg/kg of SKF-525A, 100 mg/kg of sodium phenobarbital, 50 mg/kg of aniline and 100 mg/kg of phenylbutazone, respectively. SKF-525A, aniline and sodium phenobarbital were dissolved in 0.9% NaCl solution. Phenylbutazone was dissolved into a small volume of 3N NaOH, adjusted to about pH 7.4 with 0.5N HCl and diluted with 0.9% NaCl solution.

3. Preparation of Enzymes——The method for preparing liver microsomal $9000 \times g$ supernatant fraction has been reported previously.[8]

4. Preparation of Reaction Mixtures——To a 3 ml of $9000 \times g$ supernatant fraction, 0.1 ml of $MgCl_2$ (37.5 μmole/0.1 ml for aniline hydroxylation, 50 μmole/0.1 ml for phenylbutazone hydroxylation), 0.1 ml of nicotinamide (75 μmole/0.1 ml for aniline and 100 μmole/0.1 ml for phenylbutazone), 0.1 ml (20 μmole) of glucose-6-phosphate and 0.1 ml (0.4 μmole) of NADP solution were added, and then the final volume (5.0 ml) was obtained by addition of 0.2M phosphate buffer pH 7.4. In the case of simultaneous addition of two drugs into a vial, 50 μmole of $MgCl_2$ and 100 μmole of nicotinamide were used. Reactions were carried out at 37° for 30 min aerobically.

5. Determination of *p*-Aminophenol (Estimation of Aniline Hydroxylase Activity)——The method for determining *p*-aminophenol which is formed in the incubation mixture has been described previously.[8]

6. Determination of Phenylbutazone (Estimation of Phenylbutazone Hydroxylase Activity)——The determination method, has been also described in the previous paper[9] which is a slight modification of the method of Bloxam Burns.[10,11]

Result

As the metabolic products from both aniline and phenylbutazone appear in the same incubation mixture containing substrates, the separative analysis of these products and parent substrates were examined. Analysis of the two substances did not show interference with each other.

Requirements of co-factors were checked with 1/2, 1, 2, 3 volumes of $MgCl_2$ and nicotinamide as the quantities described in the experimental. No difference of the activities was seen in any concentrations of the co-factors.

As shown in Fig. 1, the livers of rats received aniline 30 min, 12 hr and 36 hr before sacrifice showed a slightly accerelated hydroxylation of aniline. The hydroxylation of phenylbutazone was enhanced by about 40% at 30 min after administration of aniline and the activity lowered to the control level at 36 hr (Fig. 1).

No significant effects on the metabolic rates of aniline and phenylbutazone were seen by the single pretreatment of phenylbutazone (Fig. 2).

7) T. Ohshima, R. Kato and A. Takanaka, "Proceeding of the 24th Meeting of Pharmaceutical Sciences of Japan (Kyoto)," 1967, p. 326.
8) H. Kitagawa, S. Yoshida and T. Kamataki, *Yakugaku Zasshi*, **88**, 954 (1968).
9) H. Kitagawa, T. Kamataki and S. Yoshida, *Chem. Pharm. Bull.* (Tokyo), **16**, 2320 (1968).
10) D.L. Bloxam, *Biochem. Pharmacol.*, **16**, 283 (1967).
11) J.J. Burns, R.K. Rose, T. Chenkin, A. Goldman, A. Schulert and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **109**, 346 (1953).

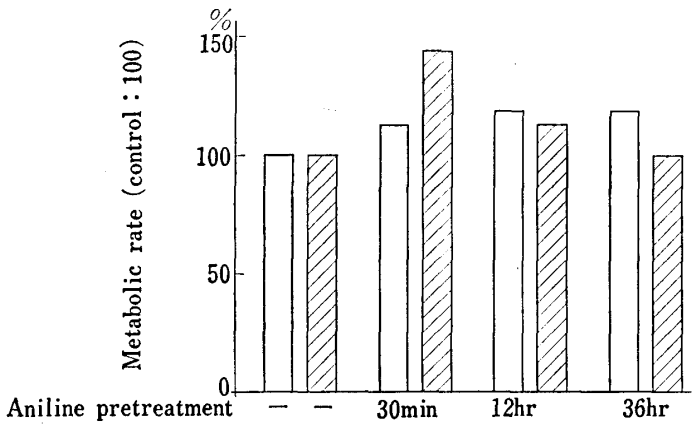

Fig. 1. Effect of Aniline (50 mg/kg *i.p.*) Pretreatment on Aniline and Phenylbutazone Hydroxylase Activities (♂)

□: aniline 3 μmole ▨: phenylbutazone 3 μmole

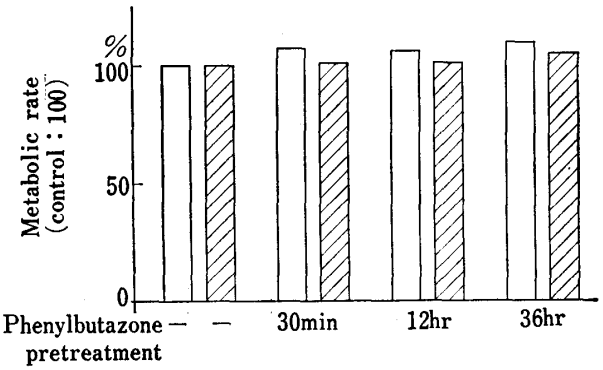

Fig. 2. Effect of Phenylbutazone (100 mg/kg *i.p.*) Pretreatment on Aniline and Phenylbutazone Hydroxylase Activities (♂)

□: aniline 3 μmole ▨: phenylbutazone 3 μmole

When the reaction mixtures containing both 1.5 μmoles of aniline and phenylbutazone were incubated, in the male rats aniline hydroxylation was diminished and phenylbutazone hydroxylation was enhanced by about 40% as compared to the activities as observed with each substrate alone. In the females, however, no variations in the metabolic velocities were found (Fig. 3).

In the case of experiments with 3 μmoles of both substrates, aniline hydroxylation was more decreased and no changes were seen in the rate of phenylbutazone hydroxylation compared with the data from the each drug alone.

Furthermore, the simultaneous addition of 3 μmoles of aniline and 1.5 μmoles of phenylbutazone produced about 20% depression of aniline hydroxylation, but the hydroxylation of phenylbutazone was increased compared to that found in the use of 1.5 μmoles of phenylbutazone alone.

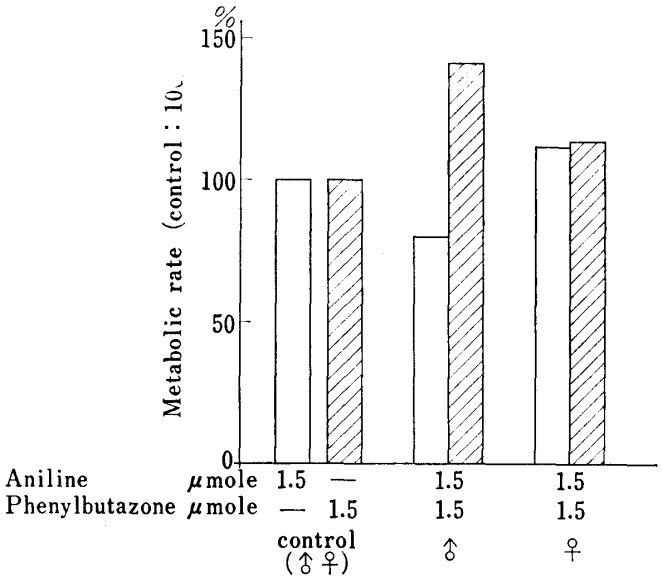

Fig. 3. Combination Effect of Aniline and Phenylbutazone on Their Hydroxylase Activities

□: aniline hydroxylation ▨: phenylbutazone hydroxylation

Moreover, simultaneous addition of 1.5 μmoles of aniline and 3 μmoles of phenylbutazone showed the considerable degree of the decreased hydroxylation of aniline and about 20% of enhanced metabolism of phenylbutazone (Fig. 4).

Phenylbutazone metabolism was significantly enhanced under the co-existence of one-twentieth fold moles of *p*-aminophenol as the moles of phenylbutazone used. As increasing the amount of *p*-aminophenol to two times as above, more increased hydroxylation of phenylbutazone occured (Fig. 5).

Livers of rats received phenobarbital 36 hr before sacrifice showed more enhanced activity of phenylbutazone hydroxylation than that of aniline hydroxylation either in the case with the substrates separately or in the combination of the two in the female rats. On the con-

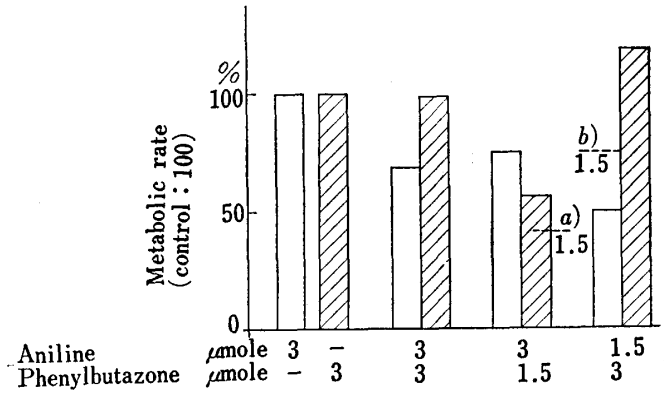

Fig. 4. Combination Effect of Aniline and Phenylbutazone on Their Hydroxylase Activities (♂)

□: aniline hydroxylation ▨: phenylbutazone hydroxylation

a) This reveals the level when 1.5 μmoles of phenylbutazone only is used as the substrate.

b) This reveals the level when 1.5 μmoles of aniline only is used as the substrate.

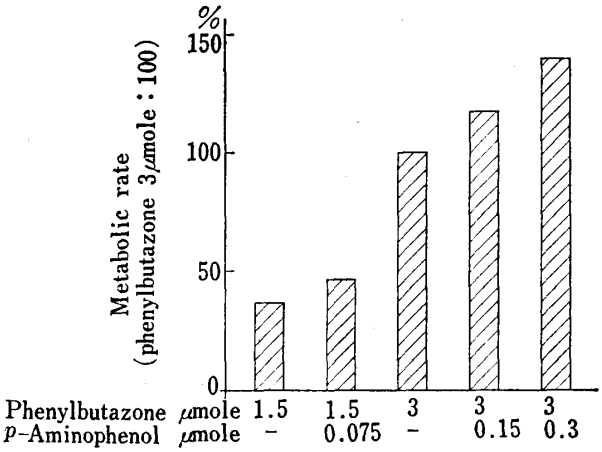

Fig. 5. Combination Effect of *p*-Aminophenol and Phenylbutazone on Phenylbutazone Hydroxylase Activity (♀)

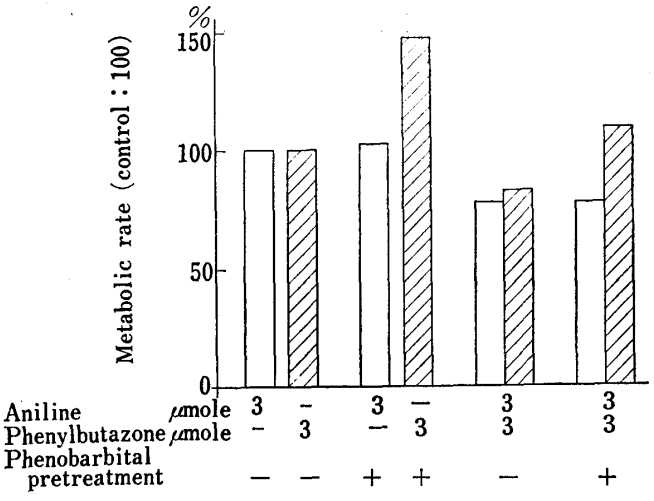

Fig. 6. Effect of Phenobarbital (100 mg/kg *i.p.* 36 hr before) Pretreatment on Aniline and Phenylbutazone Hydroxylase Activities (♀)

□: aniline hydroxylation ▨: phenylbutazone hydroxylation

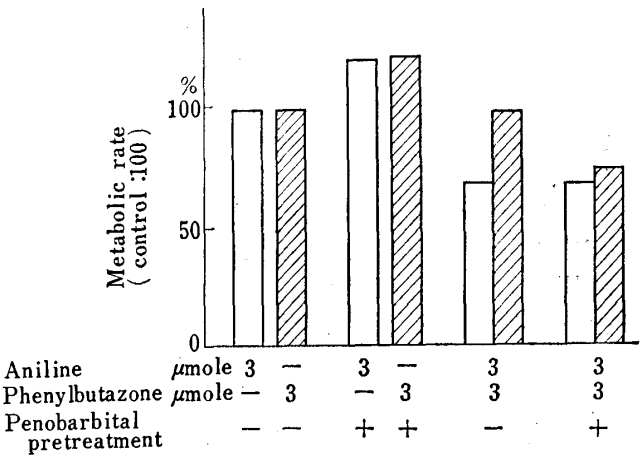

Fig. 7. Effect of Phenobarbital (100 mg/kg *i.p.* 36 hr) Pretreatment on Aniline and Phenylbutazone Hydroxylase Activities (♂)

□: aniline hydroxylation ▨: phenylbutazone hydroxylation

trary, in the male rats pretreated with phenobarbital, the decrease of the phenylbutazone hydroxylase in the combination case was observed (Fig. 6, 7). The reason why the latter phenomenon was obtained is unknown.

SKF-525A pretreatment greatly inhibited the phenylbutazone metabolism (Fig. 8, 9).

As shown in the above data, aniline hydroxylation was depressed by co-existence of phenylbutazone. Phenylbutazone hydroxylation was, however, activated by addition of either aniline or *p*-aminophenol depending upon their concentration. Moreover, the effect of phenobarbital or SKF-525A pretreatment affected stronger in the rate of phenylbutazone metabolism both in the single and combined cases.

Discussion

The evidence that accerelated metabolism of phenylbutazone occurs 30 min after giving aniline suggests that it might not due to the induced synthesis of the enzymes but is resulted from the direct effects of aniline on the enzymes or on phenylbutazone itself.

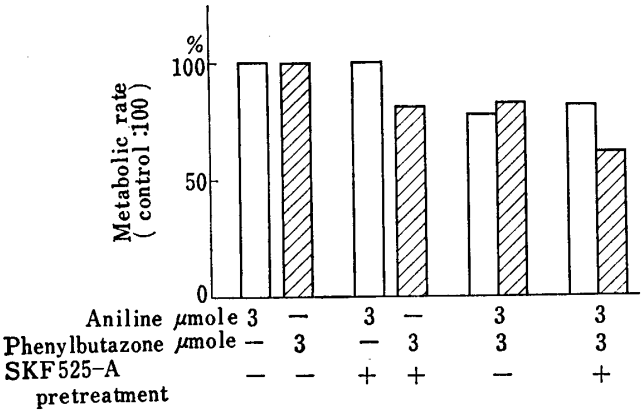

Fig. 8. Effect of SKF 525-A (40 mg/kg *i.p.* 30 min) Pretreatment on Aniline and Phenylbutazone Hydroxylase Activities (♀)

□: aniline hydroxylation ▨: phenylbutazone hydroxylation

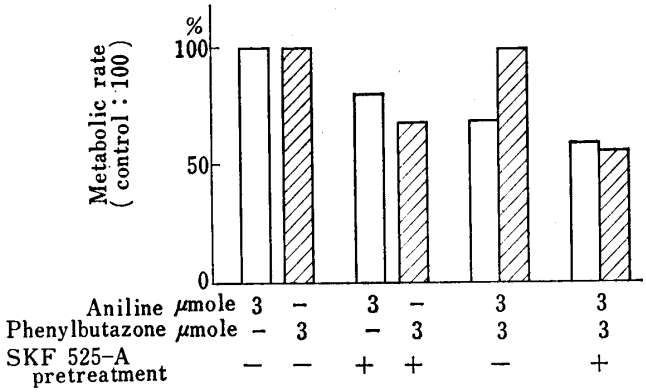

Fig. 9. Effect of SKF 525-A (40 mg/kg *i.p.* 30 min) Pretreatment on Aniline and Phenylbutazone Hydroxylase Activities (♂)

□: aniline hydroxylation ▨: phenylbutazone hydroxylation

It has been known that aniline is oxidized mainly to *o*- and *p*-aminophenol and then excreted in a conjugated form, and phenylbutazone is metabolized to the phenol in which the hydroxyl group is in the *para*-position of the benzene rings and to the (ω-1)-oxidized product of butyl side chain. In the present study, the authors determined the activity of phenylbutazone hydroxylase by measuring the disappearance of it, thus which metabolic pathway of phenylbutazone was activated by aniline is unknown.

Although many previous papers[12,13] have reported the phenylbutazone induced enzyme activities, our data revealed little induction. The induced enzyme activity might appear after repeated administration, but not be detected after only single administration as ours.

Although both aniline and *p*-aminophenol had the capacity to increase the metabolism of phenylbutazone, it is not clear as yet whether the action of these agents is due to themselves or to their metabolic products, *i.e.*, quinone. The following possibilities seemed to be reasonable.

1. Aniline or *p*-aminophenol might act as a stabilizer of phenylbutazone hydroxylase.
2. Aniline or *p*-aminophenol masks the considerable action of the inhibitor on the metabolism of phenylbutazone.
3. A possible role of aniline for the formation of P-450–phenylbutazone complex is offered, and the formed P-450–phenylbutazone-aniline complex or P-450–phenylbutazone complex might make the reduction of P-450 easier.
4. From the findings according to Y. Imai and R. Sato[14] that P-450–aniline complex enhances the reduction of P-450, aniline or *p*-aminophenol might speed up the reduction–oxidation cycle of P-450.

It has been reported that phenobabital and SKF-525A induce or inhibit the activities of the androgen sensitive enzymes.[15–18] Such effects were observed significantly in the phenylbutazone metabolism than in the aniline hydroxylation even in the combined experi-

12) B. Silvestrini, B. Catanese and P.D. Basso, *Biochem. Pharmacol.*, **15**, 249 (1966).
13) A.H. Conney, *Pharmacol. Rev.*, **19**, 317 (1967).
14) Y. Imai and R. Sato, *J. Biochem.* (Tokyo), **62**, 239 (1967).
15) H. Remmer and A.H. Conney, "Proceeding of the First International Pharmacological Meeting 6, Metabolic Factors controlling Duration of Drug Action," edited by B.B. Brodie and E.G. Erdös, Pergamon Press, 1961, pp. 235, 250.
16) L.A. Rogers, H.P. Morris and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **157**, 227 (1967).
17) R. Kato and A. Takanaka, *J. Biochem.* (Tokyo), **63**, 406 (1968).
18) R. Kato and M. Takayanagi, *Japan. J. Pharmacol.*, **16**, 380 (1966).

ments. Thus, the aniline hydroxylase may be classified as an androgen independent enzyme whereas phenylbutazone hydroxylase may be classified as an androgen dependent enzyme.

Considerable rate of metabolism of both substrates which were seen in the combined experiments suggests that these substrates may have been metabolized by different enzymes or on different sites of P-450.

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