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Inhibitory Mechanism of Imipramine on Barbiturate Metabolism in Rat Liver[1]

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Inhibition of barbiturate metabolisms by imipramine (IP) was studied systematically in the *in vitro* system and intact rats with an emphasis on the contribution of metabolism of the inhibitor itself. Both IP and its major metabolite, desmethylimipramine (DMI) were found to inhibit the *in vitro* microsomal metabolisms of various barbiturates. The time courses of the inhibitory activities of IP and DMI were also studied in the intact animals. The maximum inhibition was observed at approximately 3 hours after the administration of both IP and DMI and the significant inhibition was observed for further about 10 hours. Hexobarbital hypnosis as a pharmacological action was similarly affected by IP administration. The possible participation of the metabolite DMI was confirmed by studying the effect of preincubation of IP in the *in vitro* system and determining directly the levels of IP and DMI in the liver tissue at various times after IP administration. The effect of dose and long-term pretreatment of IP on the microsomal metabolism of barbiturates was examined and the dispositions of IP were found to be of linear nature. Species differences in the *in vitro* metabolic inhibition by IP were also examined. And furthermore, as a measure of the physiological availabilities of IP and DMI, lipid solubility, ionization constant, and absorption from the rat small intestine were discussed in connection with the inhibitory mechanism of IP.

Although several investigations[3-7] have been reported on the inhibitory effect of imipramine (IP) on the metabolisms of some drugs in the *in vitro* system and in the intact animals, in these studies, however, only apparent degree of the metabolic inhibition by IP are reported and the correlation between the inhibition characteristics and the metabolic disposition of IP has not been fully elucidated. This is true for other drug metabolism inhibitors.[8] It is now well known that many inhibitos are also metabolized alternatively in liver microsomes by the same enzyme systems responsible for drug substrates. From this point of view, investigations on the above-mentioned correlation seemed to be important. In the present studies, IP was chosen as a representative inhibitor because it is considerably metabolized both *in vitro* and *in vivo*, and it is carrying alkylamino-alkyl group which is common to the well known inhibitors of microsomal drug metabolishms.[8] The major metabolic pathway of IP in rats and man is known to be N-demethylation to desmethylimipramine (DMI), although other pathways, aromatic hydroxylation, N-oxide formation and their mixed reactions are recognized in these species.[9-11] The purpose of the present

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3) R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pharmacol.*, **12**, 357 (1963).
4) R. Kato, E. Chiesara and P. Vassanelli, *Biochem. Pharmacol.*, **13**, 69 (1964).
5) R. Pulver, B. Exer and B. Herrmann, *Arzneimittel-Forsch.*, **10**, 530 (1960).
6) F. Sjöqvist, W. Hammer, H. Schmacher and J.R. Gillette, *Biochem. Pharmacol.*, **17**, 915 (1968).
7) F. Sjöqvist and J.R. Gillette, *Life Sci.*, **4**, 1031 (1965).
8) K.J. Netter, "Proc. 1st Internat. Pharmacol. Meet.," Vol. 6, Pergamon Press, Oxford, 1962, p. 213.
9) M.H. Bickel and M. Baggiolini, *Biochem. Pharmacol.*, **15**, 1155 (1966).
10) J.V. Dingell, F. Sulser and J.R. Gillette, *J. Pharmacol. Exptl. Therap.*, **143**, 14 (1964).
11) J. Christiansen, L.F. Gram, B. Kofod and O.J. Rafaelsen, *Psychopharmacologia*, **11**, 255 (1967).

studies is to investigate systematically the significant contribution of a metabolite DMI in the inhibition of barbiturate metabolisms by IP. The evidences obtained show that DMI is also a potential inhibitor and significantly affecting the duration of the inhibition.

Experimental

Animals——Male rats of the Wistar strain weighing 90—130 g were used except the experiments in which the effect of long-term pretreatment by IP was examined with the same strain rats of both sexes. The species difference in the *in vitro* inhibition was examined with several laboratory animals of male sex other than rats. The animals were allowed free access to commercial laboratory chow and tap water at all times prior to the sacrifice.

Enzyme Preparations——The animals were killed by a sharp blow on the head, or air embolism and the liver was immediately removed and homogenized with ice-cold isotonic KCl solution in a Potter-Elvehjem type homogenizer provided with a motor-drived Teflon pestle to yield 33% (w/v) homogenate. The centrifugation of the homogenate was carried out at 9000 × g for 30 min, and the supernatant fraction was used for enzyme assays. All these tissue manipulations were performed at a temperature of 0—5°.

Enzyme Assays——The incubation mixture which is analogous to that adopted by McLuen and Fouts,[12] contained 2 ml of the supernatant fraction, 25 μmoles of glucose-6-phosphate (Sigma), 0.3 μmole of NADP (Sigma), 100 μmoles of nicotinamide, 25 μmoles of $MgSO_4$, 1.0 ml of pH 7.5 phosphate buffer (0.5 M), and 1.0 μmole of the substrate in a final volume of 5.0 ml. In the inhibition experiments, the inhibitor to be examined was added in the same incubation mixture. The mixture was incubated in a 50 ml Erlenmeyer flask for 1 hr in an air atomosphere at 37°. The enzyme activity was determined by estimating the remained substrate drug in the mixture after the incubation, and expressed in terms of μmole metabolized substrate/hr/g liver. The analytical methods for barbiturates were those reported previously; hexobarbital and N-methylallobarbital,[13] amobarbital and secobarbital,[14] and thiopental.[15]

In the experiments to study the effect of preincubation of IP on the *in vitro* hexobarbital metabolism, after various intervals of IP preincubation in the complete reaction system, the substrate was added to the mixture and incubated similarly as described above. The control metabolism in those experiments were determined by the corresponding preincubation without IP. The inhibition types and inhibitor constants of IP and DMI were determined by the method of Dixon,[16] and the Michaelis constant for hexobarbital was determined by the method of Lineweaver and Burk.[17]

In the time course study of the inhibitory effect of IP, male rats were injected *i.p.* with IP (30mg/kg), and equimolar dose of DMI (28.7 mg/kg) in saline solution (2ml/kg). The animals were sacrificed at various times after the IP administration, and the liver enzyme activity was determined by the method described above with hexobarbital and amobarbital as the substrates. The effect of IP dose was examined similarly at 3 hr after the administration by varying the dose up to 60 mg/kg. The effect of long-term pretreatment of IP was studied as follows; both sex rats were injected *i.p.* with saline solution of IP (30 mg/kg) once daily for 1, 3, and 7 days, and the enzyme activity was assayed on the first postteatment day.

Hexobarbital Hypnosis——The male rats were injected with hexobarbital (sodium salt, 100 mg/kg) *i.p.* at various times after IP administration (30 mg/kg). The duration of hypnosis was determined as usually by the righting reflex.

IP and DMI Levels in Liver——The levels of IP and DMI in the liver tissue were determined in the rat at various times after IP administration (60 mg/kg). The analytical methods for these drugs were those reported by Dingell, *et al.*[10]

Materials——Barbiturates used in the present studies were from commercially available sources and recrystallized from ethanol-water except N-methylallobarbital which was prepared by direct methylation of allobarbital by dimethyl sulfate.[18] IP and DMI were the gifts from Fujisawa Pharmaceutical Industry Ltd., Osaka. NADP and glucose-6-phosphate were purchased from Sigma Chemical Co., St Louis, Mo., U.S.A. All other chemicals were of analytical grade.

Statistics——Statistic analyses were done by the Student's t test as described by Snedecor.[19]

12) E.F. McLuen and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **131**, 7 (1961).
13) J.R. Cooper and B.B. Brodie, *J. Pharmacol. Exptl. Therap.*, **114**, 409 (1955).
14) B.B. Brodie, J.J. Burns, L.C. Mark, P.A. Lief, E. Bernstein and E.M. Papper, *J. Pharmacol. Exptl. Therap.*, **109**, 26 (1953).
15) B.B. Brodie, L.C. Mark, E.M. Papper, P.A. Lief, E. Bernstein and E.A. Rovenstine, *J. Pharmacol. Exptl. Therap.*, **98**, 85 (1950).
16) M. Dixon, *Biochem. J.*, **55**, 170 (1953).
17) H. Lineweaver and D. Burk, *J. Am. Chem. Soc.*, **56**, 658 (1934).
18) K. Kakemi, T. Arita, R. Hori and R. Konishi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1534 (1967).
19) G.W. Snedecor, "Statistical Methods," 4th ed., Iowa State College Press, Ames, 1946.

Result and Discussion

Inhibition of *in vitro* Metabolism

The inhibition of *in vitro* metabolisms of various barbiturates by IP and DMI were determined at a given inhibitor concentration (1 μmole/g liver) to compare their inhibitory activities. The results are summarized in Table I. Both the inhibitors suppressed rather uniformally *in vitro* metabolisms of all barbiturates, indicating non-specific inhibition by IP and DMI. And it is likely that a major metabolite DMI is a more potent inhibitor on barbiturate metabolisms than the parent IP. This finding is not in agreement with the conclusion of a previous

Table I. Inhibitory Activities of Imipramine (IP) and Desmethylimipramine (DMI) on *in vitro* Barbiturate Metabolisms

Barbiturates	Control metabolism[a]	Inhibition[b] (%)	
		IP	DMI
Hexobarbital	0.735	43.0	62.9
N-Methylallobarbital	0.130	41.8	55.8
Amobarbital	0.265	53.0	61.2
Secobarbital	0.314	68.2	70.9
Thiopental	0.098	56.8	61.5

a) The figures are mean values of 3—7 identical experiments with 2—6 pooled livers each, and expressed in μmole metabolized/hr/g liver.
b) The figures are mean values of 2—3 identical experiments with 2—3 pooled livers each. The amount of inhibitor added was 1 μmole per 1 g fresh liver.

work[4] which excluded DMI as a less remarkable inhibitor. The kinetic treatments have been frequently done to obtain a clue to characterize the microsomal drug enzymes in the inhibition[8,20-23] or induction studies.[24-25] In the former cases, when the inhibitor can be an alternative substrate and the metabolite(s) formed elicits considerable inhibitory activity, the observed kinetics is not attributed solely to the parent inhibitor, but to the mixed system which is complicated kinetically. In the present studies, therefore, merely as a measure of apparent affinity to the enzymatic system, the inhibitor constants of IP and DMI were determined with hexobarbital as a substrate. And it was found that the suggested type of inhibition was non-competitive for both IP and DMI, and inhibitor constants of IP and DMI were 1.7×10^{-4}M and 0.3×10^{-4}M, respectively, and they were smaller than the Michaelis constant of hexobarbital, 5.3×10^{-4}M which was estimated separately. These results suggest a possible contribution of DMI in the inhibition by IP. As an attempt to get further evidence for the DMI participation, some experiments were carried out in which IP was preincubated in the complete system before the addition of substrate. The results are shown in Table II. The inhibition observed was almost equivalent to that listed in Table I, although the control metabolisms were reduced with the lapse of time which may be due to the essential cofactor consumption. According to the quantitative estimation of IP and DMI metabolisms in the *in vitro* system similar to the present one by Bickel and Baggiolini,[9] IP was metabolized mainly to DMI (*ca.* 45% in 1 hr), on the other hand DMI was less susceptible to the susequent

20) L.A. Rogers and J.R. Fouts, *J. Pharmacol. Exptl. Therap.*, **146**, 286 (1964).
21) R. Kato and M. Takayanagi, *Jap. J. Pharmacol.*, **16**, 127 (1965).
22) M. Ikeda, S. Tanaka and T. Katayama, *Mol. Pharmacol.*, **4**, 38 (1968).
23) A. Rubin, T.R. Tephly and G.J. Mannering, *Biochem. Pharmacol.*, **13**, 1053 (1964).
24) A. Rubin, T.R. Tephly and G.J. Mannering, *Biochem. Pharmacol.*, **13**, 1007 (1964).
25) K.J. Netter and G. Seidel, *J. Pharmacol. Exptl. Therap.*, **146**, 61 (1964).

metabolism(*ca.* 9% in 1 hr). Considering these findings, it seems possible to expect that metabolically formed DMI is accumulated and significantly participate in the inhibition of barbiturate metabolisms by IP in the *in vivo* situation.

Table II. Effect of IP Preincubation on *in vitro* Hexobarbital Metabolism

Preincubation time (min)	Control metabolism (μM/hr/g)	Metabolism when IP[a] was added (μM/hr/g)		Inhibition (%)
		in preincubation	in properincubation	
45	0.430	0.228	—	47.0
45	0.430	—	0.245	43.0
60	0.323	0.182	—	43.7
60	0.323	—	0.187	42.1

a) IP was added to the system in 1.0 μmole for g fresh liver. Both incubations were done under the conditions described in methods.

Inhibition of *in vivo* Metabolism

In order to correlate the *in vivo* inhibition exerted by IP administration and the metabolic disposition of IP itself, the time courses of the inhibition of barbiturate metabolisms were studied. In these experiments, the metabolic activity of 9000×g supernatant fraction was estimated at various times after IP administration with hexobarbital and amobarbital as the substrates. The time courses of enzyme activities affected by IP are shown in Fig. 1. It was apparent from the results that IP was appreciably absorbed from the intraperitoneal route and inhibited the metabolisms of both barbiturates with the maximum effect at approximately 3hr after IP administration and the IP-produced inhibition lasted for 24 hr. This metabolic inhibition by IP was further confirmed by examining the kinetics of hypnotic action of hexobarbital. As shown in Fig. 2, the pharmacological action was influenced significantly

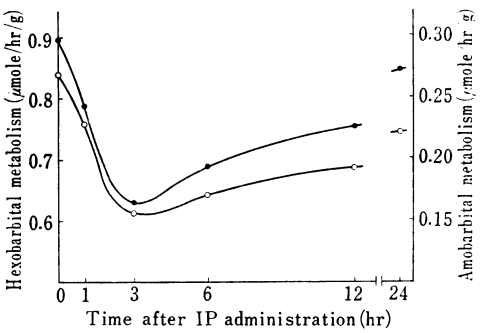

Fig. 1. Time Course of Inhibitory Activity of IP on Hexobarbital and Amobarbital Metabolisms

Each point represents the mean values of at least three rats, hexobarbital (○), amobarbital (●). Dose of IP was 30 mg/kg.

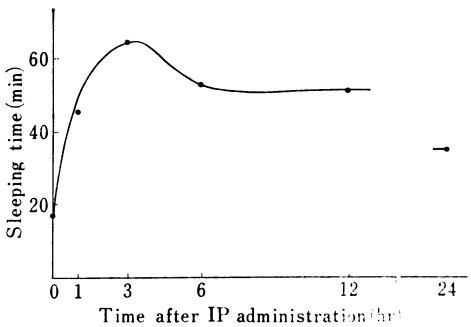

Fig. 2. Effect of IP on the Duration of Hypnotic Action of Hexobarbital

Each point represents the mean value of six rats, singnificantly different ($p<0.001$, $p<0.01$ for 24 hr) from the control sleeping time. Dose of hexobarbital was 100 mg sodium salt/kg.

by IP administration and coincided fairly well with the metabolic inhibition as shown in Fig. 1. This relationship seems to be indicative of the temporal and reversible nature of IP effect. IP has been known to have an intrinsic activity of increasing the sensitivity of the central

nervous system[3,26] in addition to its indirect effecct due to the metabolic inhibition, however considered from the recovery rate of the hypnotic potentiation and the metabolic inhibition observed, this direct effect may be relatively small. As predicted from the results of *in vitro* experiments, DMI formed *in vivo* may be an effective inhibitor on barbiturate metabolisms. According to Dingell, *et al.*,[10] the rats metabolize IP mainly to DMI more rapidly than they do DMI and the formed DMI accumulate considerably in the body. Therefore, if the pharmacokinetic parameters of DMI related to the processes of absorption and tissue distribution *etc.* are identical to those of IP, the inhibitory pattern after DMI administration will be similar to that observed after IP administration, although some deviations will be observed probably due to the different metabolic dispositions of IP and DMI. On this line of discussion, the time courses of the inhibition of barbiturate metabolisms elicited after DMI administration were examined. The results shown in Fig. 3 seem to be consistent with the above-mentioned trends. These findings suggest that the concentration of IP and its metabolite DMI present in liver cells around the microenvironment of the responsible microsomal enzymes is necessary for the effective inhibition. Further experiments were performed concerning with the effect of dose of IP on the enhancement of the inhibition. The results are shown in Fig. 4. For both the

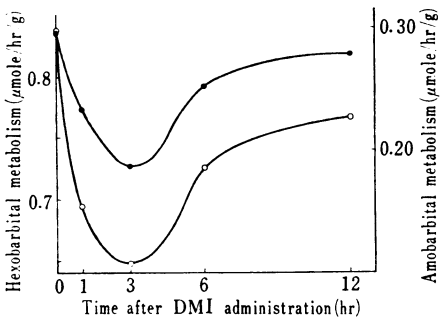

Fig. 3. Time Course of Inhibitory Activity of DMI on Hexobarbital and Amobarbital Metabolisms

Each point represents the mean value of at least three rats, hexobarbital (○), amobarbital (●). Dose of DMI was 28.7 mg/kg equimolar to 30 mg/kg of IP.

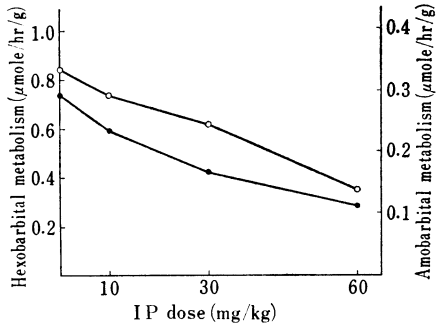

Fig. 4. Effect of IP Dose on Hexobarbital and Amobarbital Metabolisms

Each point represents the mean value of at least five rats, hexobarbital (○), amobarbital (●). Enzyme assays were done at 3 hr after IP administration.

substrates, the inhibitory effect of IP was found to be dose-dependent within the range examined, although the enhancement was not strictly linear. It can be assumed kinetically that the increase in dose will result in an increase in the effective concentration of the drug or its metabolite and the corresponding inhibition will be also increased if the physiological dispositions are of linear and passive nature. The present situation seems to be the case. The dose-dependent inhibition, however, has a possibility that it is associated with the alterations of the hepatic cell ultrastructure which are known to be causative of the change in drug metabolisms.[27] An attempt, therefore, was made to examine the effect of long-term pretreatment with IP on the enzyme activity as a possible criterion for the ultrastructural alterations. As shown in Fig. 5, any significant effect was not observed by the chronic treatment in both male and female rats. From the results obtained hitherto, it seems possible to assert that the concentration of IP and DMI present in the liver cells is an essentially

26) I. McCance, *Arch. Intern. Pharmacodyn.*, **148**, 270 (1964).
27) H. Remmer and H.J. Merker, "Proc. 2nd Intern. Pharmacol. Meet.," Vol. 4, Pergamon Press, Oxford, 1965, p. 299.

important factor in determining the extent of the inhibition and the concentration changes of these compounds with the lapse of time is related to the duration of the inhibitory effect after IP administration. Based on this reasoning, the liver levels of IP and DMI were determined after IP administration in male rats. The results represented in Fig. 6 offers a plausible explanation for underlying mechanism of the inhibition suggested above.

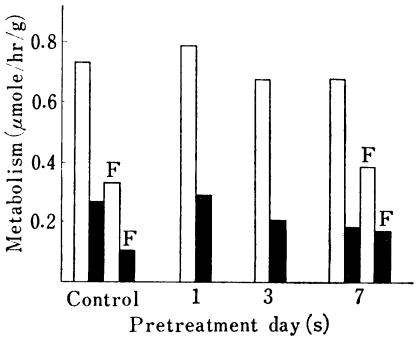

Fig. 5. Effect of Long-Term Pretreatment of IP on Hexobarbital and Amobarbital Metabolisms

Each bar represents the mean value of at least three rats. Enzyme assays were done at 24 hr after the last injection. Dose of IP was 30 mg/kg *i.p.* once daily.
□: hexobarbital
■: amobarbital
F: female rat

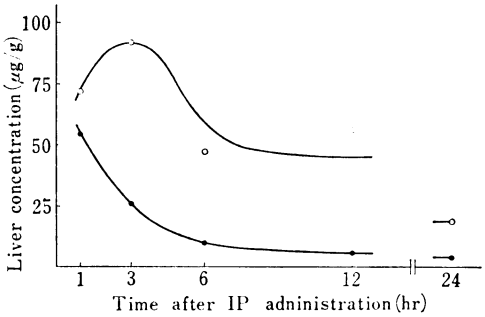

Fig. 6. Liver Concentrations of IP and DMI after a Single Dose of IP

Each point represents the mean value of two experiments, IP (●), DMI (○). Dose of IP was 60 mg/kg.

TABLE III. Species Difference of Inhibitory Activity of IP and DMI on *in vitro* Hexobarbital and Amobarbital Metabolism

Animal species[a]	Hexobarbital		Amobarbital	
	IP	DMI	IP	DMI
Rat	13.5[b]	33.6	26.9	56.6
Mouse	31.8	58.9	23.1	53.0
Rabbit	52.6	57.9	51.4	79.2
Guinea pig	13.2	15.3	14.2	12.2

a) Male adult animals were used and enzyme preparations were same as in rats described in Methods.
b) The figures are % inhibition and mean values of at least 3 experiments. The amount of inhibitor added was 0.2 μmole for g fresh liver.

The inhibitory activities of IP and DMI for *in vitro* barbiturate metabolisms were examined further in other animal species. As shown in Table III, in all species DMI was also active as an inhibitor. Because it is known that there is a marked species differences in the metabolic dispositions of IP,[10] these data can not be correlated with those in the *in vivo* studies which require further investigations.

Physiological Availability of IP and DMI

As the physicochemical properties affecting the physiological availability of a drug in the body, ionization constant and lipid solubility of drug molecule are often discussed.[28]

28) L.S. Schanker, *Ann. Rev. Pharmacol.*, **1**, 29 (1961).

The previously reported pK_a values of IP and DMI are in the ranges of 7.94—9.5 and 9.22—10.2, respectively.[29-30] The molecular species of IP and DMI at physiological pH are predominantly in the charged formed, but the apparent partition coefficients of IP and DMI, determined with the pH 7.4 isotonic phosphate buffer–chloroform system at 37° were 1925 and 453, respectively. Considering the generalized theory of membrane permeation, these data suggest that IP and DMI are almost equivalently available in the absorption and tissue distribution processes.[31]

Further studies on the molecular-level correlation of drug enzyme inhibition and the inhibitor's dispositions with the other inhibitors should be of interest and required to chracterize the microsomal drug metabolizing enzyme systems.

29) R.A. Maxwell, P.D. Keenan, E. Chaplin, B. Roth and S.B. Eckhardt, *J. Pharmacol. Exptl. Therap.*, **166**, 320 (1969).
30) M.H. Bickel and H.J. Weder, *J. Pharm. Pharmacol.*, **21**, 160 (1969).
31) The absorption rate constants of IP and DMI from rat small intestine were determined and the value 1.43 hr^{-1} was obtained for both the inhibitors.