Biochemical Pharmacology, Vol. 19, pp. 1363–1369. Pergamon Press. 1970. Printed in Great Britain

STUDIES ON THE DEMETHYLATION, HYDROXYLATION AND *N*-OXIDATION OF IMIPRAMINE IN RAT LIVER*

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(*Received* 7 *June* 1969; *accepted* 29 *August* 1969)

Abstract—Metabolic pathways responsible for the demethylation, *N*-oxidation and 2-hydroxylation of imipramine were studied in rat liver *in vitro*. Isotope-trapping experiments have revealed that imipramine-*N*-oxide is not involved as an intermediate in the formation of desmethylimipramine from imipramine. The enzyme systems for demethylation and 2-hydroxylation are induced by phenobarbital, but that for *N*-oxidation is not induced. All available evidence indicates that the demethylation, *N*-oxidation and 2-hydroxylation reactions may be independent of one another.

IMIPRAMINE, *N*-(3-dimethylaminopropyl)iminodibenzyl hydrochloride, is a clinically effective antidepressant agent, but the mechanism of action is still far from being understood. In 1960, Herrmann and Pulver[1] isolated 2-hydroxyimipramine, its glucuronide, and desmethylimipramine from the urine of a patient treated with imipramine. Subsequently, seven additional metabolites derived from this drug, including imipramine-*N*-oxide, have been isolated and identified in human urine.[2–5] The metabolism of imipramine *in vitro* was studied first by Dingell *et al.*,[6] who showed that this drug might be degraded by a rat liver microsomal enzyme system which required $NADPH_2$ and oxygen. Bickel and Baggiolini[7] also attempted to elucidate the metabolism of imipramine with rat liver *in vitro* and suggested that the drug may be metabolized by three independent pathways which included *N*-oxidation, demethylation and 2-hydroxylation reactions, as shown in Fig. 1. McMahon and Sullivan[8] and Kuntzman *et al.*[9] studied the metabolisms of tertiary amines such as 1-propoxyphene and chlorcyclizine and proposed that the *N*-oxidation reaction in general may be independent of the demethylation reaction. However, Ziegler and Pettit[10] have shown that dimethylaniline is converted to monomethylaniline by pork liver microsomes through two consecutive reactions, *N*-oxidation to dimethylaniline-*N*-oxide followed by dealkylation of the latter to monomethylaniline. Recently, Bickel *et al.*[11] re-examined the previous report and suggested that imipramine-*N*-oxide might be reduced to imipramine and also to a minor degree demethylated to desmethylimipramine in various tissues, and that these reactions may also proceed non-enzymically in the presence of either heme or a metal–chelate complex.

The present studies were undertaken, therefore, with an isotope-trapping technique to re-examine a possible relation between the *N*-oxidation and demethylation reactions of imipramine in rat liver, and further, to compare the involvement of these possible pathways in imipramine metabolism. The results to be presented indicate that imi-

* This investigation was supported in part by the Scientific Research Fund of Fujisawa Pharmaceutical Co., Ltd.

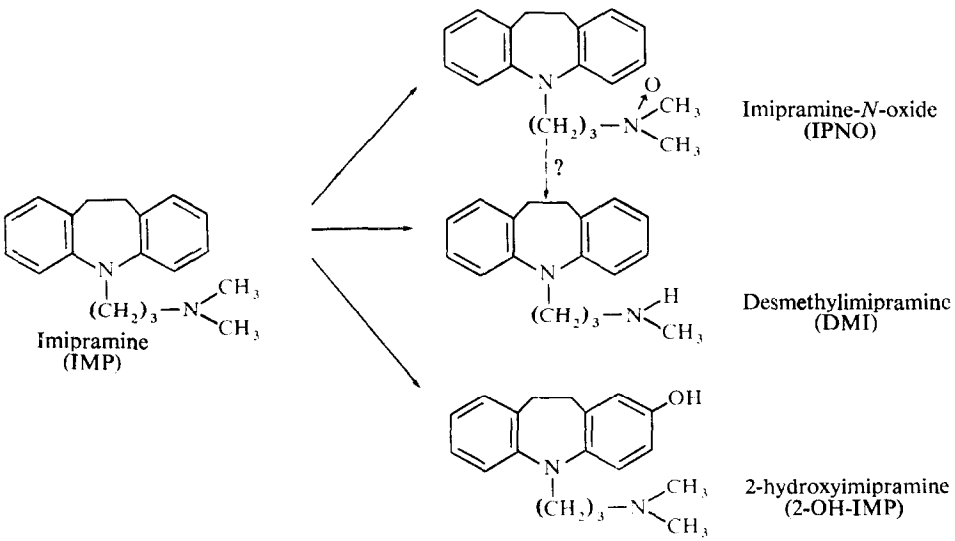

FIG. 1. Metabolic pathways of imipramine.

pramine-*N*-oxide is not an intermediate in the formation of desmethylimipramine from imipramine. The properties of enzymes responsible for the *N*-oxidation, demethylation and 2-hydroxylation of imipramine are also briefly presented.

MATERIALS AND METHODS

Materials. Imipramine-^{14}C, *N*-(3-dimethylaminopropyl)iminodibenzyl(methylene-^{14}C)hydrochloride, was purchased from the Radiochemical Centre, Amersham, Buckinghamshire, England. Glucose 6-phosphate, NADP and glucose 6-phosphate dehydrogenase (EC 1.1.1.49) were obtained from the Sigma Chemical Company. Imipramine and the authentic samples of its metabolites were gifts from Dr. K. Scheibli, J. R. Geigy S. A., Basel, Switzerland. SKF 525A(β-diethylaminoethyldiphenylpropyl-acetate HCl), SKF 8742A(β-monoethylaminoethyldiphenylpropyl-acetate HCl) and AEDV(aminoethyl-2,2-diphenyl valerate HCl) were donated by Dr. M. Ikeda, Kyoto University, Faculty of Medicine, Japan.

Experimental methods. Male Wistar rats, weighing 150–200 g, normally fed *ad lib.* with CLEA CE2 laboratory Chow obtained from the Japanese Central Laboratory Experiment Animals Company, were used. The rats were sacrificed by exsanguination followed by decapitation. The livers were quickly removed, chilled in an ice-bath, and homogenized with 10 vol. of 0·25 M sucrose in a Potter–Elvehjem glass-Teflon homogenizer. The homogenates were centrifuged for 20 min at 0–5° at 9000 *g*. The postmitochondrial supernatant thus obtained contained 0·11 g protein per g of liver and was employed as the enzyme source in the present studies.

Unless otherwise specified, the standard reaction mixture contained: 65 mμmoles imipramine-^{14}C (4800 cpm/mμmole); 1 μmole glucose 6-phosphate; 100 mμmoles NADP; 1 unit glucose 6-phosphate dehydrogenase; 1 μmole $MgCl_2$; 100 mμmoles nicotinamide; 25 μmoles Tris-HCl, pH 7·4; and 0·03 ml (0·3 mg protein) of the

postmitochondrial supernatant in a total volume of 0·45 ml. The mixture was incubated for 25 min at 37°. The reaction was stopped by heating the mixture for 1 min in a boiling water bath. After centrifugation, a 0·03 to 0·05-ml aliquot of the supernatant was chromatographed on Whatman No. 3 paper, previously immersed in 10% peanut oil in acetone, using a solvent system of 28% ammonia–methanol (1:1).[12] Authentic samples of various possible metabolites were cochromatographed. The spots were visualized by spraying the paper with the following diazo reagent: a mixture of 0·5 g *p*-nitroaniline in 50 ml of 1 N HCl, 0·5 g $NaNO_2$ in 50 ml water, and 0·5 g sulfanilic acid in 50 ml water (1:1:1), followed by an additional spray of concentrated HCl.[7] The radioactivity on paper was determined by direct paper strip counting using a Packard Tri-Carb liquid scintillation spectrometer, with 0·5% 2,5-diphenyloxazole and 0·03% 1,4-bis[2-(5-phenyloxazolyl)] benzene in toluene as a scintillator.

Protein was determined by the method of Lowry *et al.*[13]

RESULTS AND DISCUSSION

Imipramine-^{14}C was incubated with the postmitochondrial supernatant of rat liver under the conditions described in Materials and Methods. When an aliquot of the reaction mixture was chromatographed on paper, three major radioactive products were formed, as shown in Fig. 2. These products were identified as desmethylimipramine, 2-hydroxyimipramine and imipramine-*N*-oxide by paper chromatography in three different solvent systems. Two additional minor radioactive products were produced and, although they have not yet been identified, neither one has paper chromatographic properties corresponding to those of either 2-hydroxyimipramine

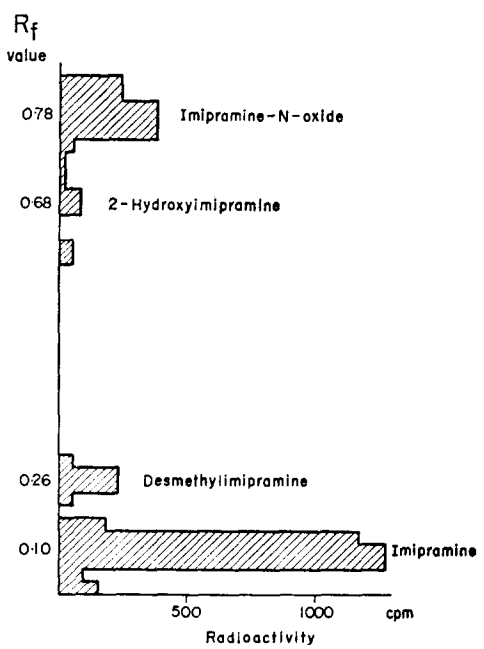

FIG. 2. Isolation of imipramine and its metabolites by paper chromatography. The standard reaction mixture was incubated as described in Materials and Methods. A 0·03-ml aliquot of the mixture was chromatographed as described in the text.

glucuronide or 2-hydroxydesmethylimipramine. Recently, Bickel and Weder[14] have identified fourteen metabolites of imipramine in rats. Perhaps the use of the chromatographic systems of these authors with authentic samples would allow the resolution of these two spots.

Bickel and Baggiolini[7] reported that the optimal concentration of $MgCl_2$ for the demethylation reaction was $1{\cdot}8 \times 10^{-2}$ M. The effect of various concentrations of magnesium ion on these reactions of imipramine metabolism was re-examined and the results are shown in Fig. 3. The optimal concentration of this ion for the demethylation reaction was 2×10^{-3} M. This discrepancy is probably due to the fact that the buffer solution employed by Bickel and Baggiolini[7] contained ethylenediaminetetraacetate in a final concentration of 6×10^{-3} M. The imipramine-*N*-oxide formation

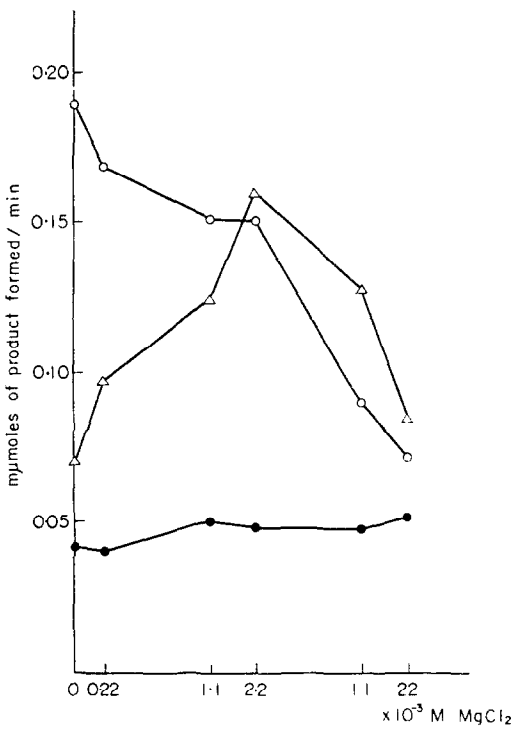

FIG. 3. The effect of $MgCl_2$ on the demethylation, *N*-oxidation and 2-hydroxylation reactions of imipramine. The standard assay system was used with $MgCl_2$ as indicated. Imipramine-*N*-oxide, ○—○; desmethylimipramine, △—△; 2-hydroxyimipramine, ●—●.

was inhibited with increasing concentrations of magnesium ion, while the formation of 2-hydroxyimipramine appeared to be independent of this ion in the range of 0·22 to 22×10^{-3} M.

In order to ascertain whether imipramine-*N*-oxide is involved as an intermediate in the formation of desmethylimipramine from imipramine, imipramine-^{14}C was incubated under the standard conditions with various concentrations of nonradioactive imipramine-*N*-oxide as a co-substrate. After the reaction was ended, the

TABLE 1. THE ISOTOPE-TRAPPING EXPERIMENT WITH NONRADIOACTIVE IMIPRAMINE-*N*-OXIDE AS A CO-SUBSTRATE*

	Radioactive metabolites formed (cpm/mg protein)			
	Nonradioactive imipramine-*N*-oxide added			
	none	10^{-3} M	2×10^{-4} M	2×10^{-5} M
Desmethylimipramine	2240	1890	2540	2870
Imipramine-*N*-oxide	4080	4290	4440	3370

* The standard assay system was used except that different concentrations of nonradioactive imipramine-*N*-oxide were added.

desmethylimipramine and imipramine-*N*-oxide were reisolated from the reaction mixture. As shown in Table 1, no additional radioactivity was trapped in the imipramine-*N*-oxide fraction and practically the same quantities of desmethylimipramine were produced in the presence and absence of the co-substrate. The specific activity of the desmethylimipramine produced was practically identical to that of the imipramine employed as a substrate. Under the experimental conditions, no significant quantity of desmethylimipramine was produced from imipramine-*N*-oxide either enzymically or nonenzymically. The results may indicate that imipramine-*N*-oxide is not involved in the demethylation of imipramine.

Further evidence for this conclusion was provided by the fact that the enzyme systems responded to phenobarbital in different manners. Young male rats (50 g) were given an intraperitoneal injection of sodium phenobarbital (75 mg/kg) daily for 4 days, and the enzymic production of desmethylimipramine, 2-hydroxyimipramine and imipramine-*N*-oxide was determined. The results are summarized in Table 2. The enzyme activities responsible for the demethylation and 2-hydroxylation reactions were stimulated 2-fold. In contrast, the enzyme system for the *N*-oxidation reaction was not induced. The results suggest that the *N*-oxidation reaction is due to an enzyme system different from that employed for the demethylation and 2-hydroxylation reactions in imipramine metabolism.

The sensitivities to inhibitors such as SKF 525A corroborated the difference between the three reactions. As shown in Fig. 4, the *N*-oxidation reaction was relatively insensitive, but the demethylation reaction was very sensitive to SKF 525A, SKF 8742A and

TABLE 2. EFFECT OF PHENOBARBITAL TREATMENT ON THE DEMETHYLATION, HYDROXYLATION AND *N*-OXIDATION REACTIONS OF IMIPRAMINE*

	Metabolites of imipramine formed (mμmoles/min/mg protein)	
	Control	Phenobarbital
Desmethylimipramine	0·297 ± 0·048†	0·683 ± 0·193
2-Hydroxyimipramine	0·153 ± 0·018	0·303 ± 0·038
Imipramine-*N*-oxide	0·507 ± 0·078	0·510 ± 0·084

* Immature male rats (50 g) were injected i.p. with sodium phenobarbital (75 mg/kg) daily for 4 days.
† Mean ± S. D.

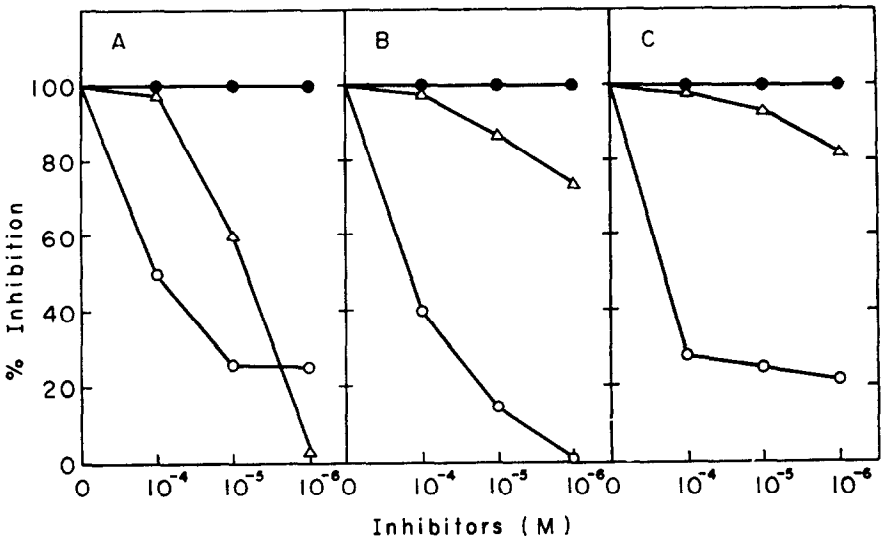

FIG. 4. Effects of SKF 525A, SKF 8742A and AEDV on the demethylation, *N*-oxidation and 2-hydroxylation reactions of imipramine. The standard assay system was used except that the inhibitors were added in a final concentration of 10^{-4} to 10^{-6} M. Imipramine-*N*-oxide, ○—○; desmethylimipramine, △—△; 2-hydroxyimipramine, ●—●. (A) SKF 525A; (B) SKF 8742; (C) AEDV.

AEDV used in final concentrations of 10^{-4} to 10^{-6} M. The results are consistent with the observation by Ziegler and Pettit[10] that SKF 525A and carbon monoxide do not inhibit the *N*-oxidation of dimethylaniline.

Under certain conditions, *N*-oxides of tertiary amines have been shown to react with ferrous ion to yield the demethylated and reduced derivatives.[9, 15] The nonenzymic demethylation and reduction of imipramine-*N*-oxide were confirmed by Bickel *et al.*[11] However, an enzyme system has not been detected thus far which converts imipramine-*N*-oxide directly to desmethylimipramine. The hydroxylation of position 2 of imipramine is also induced by phenobarbital, but may be distinguished clearly from the other two reactions, since it is almost completely inhibited by SKF 525A, SKF 8742A and AEDV.

The postmitochondrial supernatant was incubated for 15 min with various concentrations ($8{\cdot}9 \times 10^{-6}$ to $3{\cdot}3 \times 10^{-5}$ M) of imipramine-^{14}C under the standard conditions, and the apparent K_m values for the three reactions used for imipramine metabolism were determined according to the method of Lineweaver and Burk.[16] Under these conditions, the reactions proceeded linearly with time. When each determination was made with three separate runs with the same pool of the enzyme solution, the apparent K_m in the 2-hydroxylation reaction was found to be 5×10^{-4} M, somewhat greater than that (2×10^{-4} M) of both the demethylation and *N*-oxidation reactions.

The results presented in this paper indicate that imipramine-*N*-oxide is not involved as an intermediate in the enzymic formation of desmethylimipramine and that the metabolic pathways of imipramine caused by demethylation, 2-hydroxylation and *N*-oxidation may be independent of one another. Hence, some unexpected clinical effects of imipramine[17–19] may be the results of altered metabolic activities of a

selected enzyme responsible for the biotransformation of this drug. Although Brodie *et al.*[20] have proposed a generalized mechanism for the dealkylation reaction, which involves the hydroxylation of the alkyl group, the precise mechanism of the demethylation of imipramine will be explored by further investigations.

Acknowledgements—I am grateful to Dr. M. Ikeda and Dr. H. Kawai of the Faculty of Medicine, Kyoto University, for valuable discussions. I am indebted to Dr. Y. Nishizuka, Kobe University School of Medicine, for his critical reading of this manuscript. The technical assistance of Miss S. Nishimura is gratefully acknowledged.

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