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THE METABOLISM OF IMIPRAMINE AND ITS METABOLITES BY RAT LIVER MICROSOMES*

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Abstract—The metabolic pathways of imipramine and its metabolites by rat liver microsomes have been studied with the aim to obtain a comprehensive picture of the metabolism of imipramine and further insight into the action of the microsomal drug-metabolizing enzyme system. Paper- and thin-layer chromatography was used for detection and determination of metabolites; demethylation was additionally determined by formaldehyde assay.

Imipramine is extensively metabolized and in addition to the known major metabolites, desmethylimipramine and 2-hydroxy-imipramine, all ten hitherto described metabolites of imipramine were found to be formed in the rat, nine of them *in vitro*. Time course-studies and the separate investigation of the metabolism of imipramine metabolites allow conclusions as to the sequence of metabolite formation, and primary, secondary and tertiary metabolites of imipramine can be distinguished. A total of sixteen metabolic pathways were detected: demethylations, aromatic hydroxylations, side chain dealkylations, N-oxidation, N-oxide reduction and conjugations. Based on these results an attempt was made to establish a comprehensive scheme of the metabolism of imipramine in the rat and to estimate relative conversion rates for the metabolic pathways.

STUDIES on the metabolic fate of imipramine have been published soon after the introduction of the drug into the therapy of mental depression. In 1960 Schindler[1] reported on 2-hydroxy-imipramine, the first metabolite isolated. Herrmann and Pulver[2] discovered the glucuronide of this metabolite in addition to desmethylimipramine. Herrmann, *et. al.* in the same year reported on three additional metabolites: Desdimethylimipramine, 2-hydroxy-desmethylimipramine and its glucuronide.

Pharmacological studies by Sulser, *et. al.* showed that the anti-depressant (anti-reserpine) activity of imipramine in rats is mediated by the formation *in vivo* of desmethylimipramine. This finding received additional evidence by the accumulation in rat brain of desmethylimipramine after imipramine treatment of the animals as shown by Gillette *et al.*[5] Using methyl-^{14}C-labelled imipramine in the rat Bernhard and Beer[6] were able to confirm demethylation of the drug. The pharmacological profile of desmethylimipramine has been compared to that of its parent compound by Brodie, *et. al.*[7, 8].

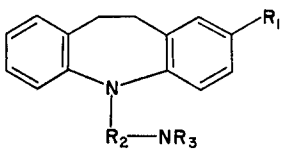
Finally the following minor metabolites of imipramine have been detected: imipramine-N-oxide[9], iminodibenzyl and its 2-hydroxy derivative[10] and — without proof —2-hydroxy-desdimethylimipramine[11].

* Abbreviations used: G-6-P = glucose-6-phosphate; G-6-P-dh = glucose-6-phosphate dehydrogenase, PP = pyrophosphate; TEA = triethanolamine; EDTA = ethylenediamine tetraacetic acid.

A major contribution to our knowledge of the metabolism of imipramine and desmethyl-imipramine (desipramine, DMI) was made by Dingell, Sulser and Gillette.[12,13] These authors compared metabolism *in vivo* and *in vitro* in different species, relating the species differences in metabolism to those in pharmacological action. In addition they found that metabolism of imipramine occurs only in liver and is due to the action of the microsomal enzyme system ("drug enzymes") requiring $NADPH_2$ and O_2.

As a result of these preceding investigations ten metabolites of imipramine are known (Table 1), showing that the microsomal enzyme system must be regarded as a

TABLE 1. IMIPRAMINE AND ITS METABOLITES



Symbol*	Compound	R_1	R_2	R_3	Species and references
IP	Imipramine	H	$(CH_2)_3$	CH_3, CH_3	
DMI	Desmethyl-imipramine	H	$(CH_2)_3$	CH_3, H	rabbit[2,3,13], rat[3,5,13] human[2,11,13]
DDMI	Desdimethyl-imipramine	H	$(CH_2)_3$	H, H	rabbit[3], rat[3,13], human[11]
IPNO	Imipramine-N-oxide	H	$(CH_2)_3$	CH_3, CH_3O	human[9,11]
IDB	Iminodibenzyl	H	H	—	human[10]
OH-IP	2-hydroxy-imipramine	OH	$(CH_2)_3$	CH_3, CH_3	rabbit[2,3], rat[13], human[1,2,11]
OH-DMI	2-hydroxy-desmethyl-imipramine	OH	$(CH_2)_3$	CH_3, H	rabbit[3], human[11]
OH-DDMI	2-hydroxydesdimethyl-imipramine	OH	$(CH_2)_3$	H, H	[11]
OH-IDB	2-hydroxyiminodibenzyl	OH	H	—	human[10]
OH-IP-GA	2-hydroxy-imipramine glucuronide	O-GA†	$(CH_2)_3$	CH_3, CH_3	rabbit[2]
OH-DMI-GA	2-hydroxydesmethyl-imipramine-glucuronide	O-GA†	$(CH_2)_3$	CH_3, H	rabbit[3]

* As used in this paper.
† GA = glucuronic acid.

multi-enzyme system. However, the existence of these metabolites and their rates of formation were elucidated from studies with different systems under different conditions and the use of different species. Furthermore only the major metabolites have been investigated.

The aim of this study was to obtain a comprehensive picture of the metabolism of imipramine in one species, i.e. to follow qualitatively and quantitatively the multiple actions of the microsomal enzymes upon the substrate under investigation. Time-course studies and a separate investigation of the biotransformation of the imipramine metabolites were expected to shed light on biochemical problems involved in multiple metabolite formation such as the sequence and rates of reactions (primary and secondary metabolites) and the course of the pathways used.

A strict limitation to a single, well defined system operated under constant conditions was imperative in order to serve the purpose of this investigation. The rat was chosen as species because this animal seems to be closest to humans in regard to imipramine metabolism.[13] The metabolic fate of imipramine and of its metabolites was studied *in vitro* using liver microsomal enzyme preparations and cofactors. Paper- and thin-layer chromatography methods were developed for the detection of imipramine and all its metabolites. Furthermore, demethylation—the major metabolic pathway of imipramine—was accounted for by determination of formaldehyde formed. The main investigation was preceded by a series of systematic experiments with the *in vitro* system. These experiments were intended to serve two separate purposes: (1) to find out optimal conditions for imipramine metabolism, i.e. to establish a standardized incubation procedure to be used in all experiments of the main investigation and (2) to test the influence of various components of the incubation system and of experimental conditions on enzymatic activity.

MATERIALS AND METHODS

Liver microsomal preparation

Male Wistar rats weighing 200–250 g and normally fed were used. The chilled livers were homogenized for 1 min in a Potter-Elvehjem homogenizer with 3 ml of buffer solution. The homogenates were centrifuged for 30 min at 9000 *g* in an MSE cold centrifuge to remove nuclei and mitochondria. The 9000 *g*-supernatant was used as such or was centrifuged 60 min at 100,000 *g* in a preparative Spinco ultracentrifuge. The microsomal precipitate was resuspended in buffer and re-centrifuged for 60 min at 100,000 *g*. All operations were carried out between 0 and 5°.

Microsomal preparations corresponding to 250 mg liver were used for the incubations. The protein contents were assayed with the biuret method.

Standard incubation mixture

Unless otherwise stated the following incubation mixture was used: 9000 *g*-supernatant or microsome suspension corresponding to 250 mg liver, 2 μmoles-substrate, 50 μmoles glucose-6-phosphate, 1·0 μmolesNADP, 1·3 I.U. G-6-P-dehydrogenase, 100 μmoles niacin, 90 μmoles $MgCl_2$, 60 μmoles semicarbazide hydrochloride and triethanolamine-HCl-EDTA buffer[14] 0·09 M, pH 7·5 to a total volume of 5 ml. Only analytical grade reagents were used. Scintillation vials (Packard Instrument Co.) were used as incubation vessels. The incubations were started by the addition of microsomal enzyme preparation and run on a metabolic shaker under air at 37°.

Measurement of formaldehyde

Formaldehyde formed was measured by the colorimetric method of Nash[15] as modified by Cochin and Axelrod.[16] A 3·5 per cent borax solution was used instead of $Ba(OH)_2$ for protein precipitation. The yellow chromophore formed by the Nash reaction was extracted in water-saturated iso-amylalcohol.[17]

Chromatography

For paper chromatography the method described by Herrmann in 1963[11] was used. The two solvents allow for separation of the basic and the phenolic metabolites

respectively. The spots were visualized by spraying the paper with the following diazo reagent: 0·5 g *p*-nitroaniline in 50 ml 1 N HCl + 0·5 g $NaNO_2$ in 50 ml water + 0·5 g sulfanilic acid in 50 ml water. An additional spray of conc. HCl was needed.

For quantitative evaluation the blue spots (basic metabolites and IPNO) were eluted in 4 ml conc. HCl/methanol 1:1 for 1 hr and the extinction of the filtered eluate measured with a filter photometer at 578 nm. The red spots (phenolic metabolites) were eluted with 4 ml methanol and the extinction measured at 496 nm.

Better and more rapid separation was obtained by the use of thin layer chromatography on Silica Gel G of 0·3 mm thickness. Imipramine and all its unconjugated metabolites could be separated in one run with one solvent: chloroform/propanol/sat. ammonia 100: 100: 2. The same spray as with paper chromatography was used. In a few instances the solvent described by Im Obersteg and Bäumler[10] was also used Quantitative evaluation of thin layer chromatography was done by the method of Pelka and Metcalfe[18], based on a linear relationship between the logarithm of the amount of substance and the square root of the area of the spot.

Extraction of imipramine and metabolites

Immediately after the incubation the contents of the vessels were brought to pH 9 with NaOH and extracted with 1·2-dichlorethane for 30 min. The organic phases were evaporated to dryness under reduced pressure and the residues taken up in 0·5 or 0·3 methanol. Aliquots of 2–30 μl were used for chromatography.

For determination of the conjugated metabolites the aqueous phase was incubated at pH 5·4 with a mixture of 1 I.U. β-glucuronidase and 0·5 I.U. aryl-sulfatase for 24 hr at 37°. The incubation mixture was then extracted with 1·2-dichlorethane at pH 9 and treated like above.

Partition coefficients

5 mg of the substance was distributed by shaking with 5 ml phosphate buffer pH 7·5 (0·15 M) and chloroform for 30 min at 20°. 10 μl samples were then run on thin layer plates. On one chromatogram the phase containing less material was run in a suitable concentration range (e.g. concentrated 20-fold) together with equal volumes of a dilution series of the other phase (e.g. dilutions 1, 2, 4, 8, 16, 32). This procedure yielded partition coefficients of considerable accuracy.

RESULTS

Testing of the incubation system

Imipramine was incubated for 1 hr at 37° under air. Unless otherwise stated suspensions of microsomal fraction (100,000 *g* pellet) was used as enzyme preparation. The concentration of enzyme preparation was determined by protein assay (biuret method). Enzymatic activity was measured in terms of demethylation, i.e. of formaldehyde formation.

Increase in the concentrations of the $NADPH_2$-generating cofactors, G-6-P and G-6-P-dh, resulted in a more rapid demethylation within the tested range (Fig. 1).

The following buffer solutions have been tested: PP-KCl: Pyrophosphate/KCl 1·15 per cent (v/v = 1/1),[19] Collidine,[20] TRIS,[21] TEA: Triethanolamine-HCl-EDTA.[14] In each case the pH was 7·5 and the molarity 0·15. three ml were used per 5 ml of incubation mixture. Phosphate buffer has been excluded because G-6-P-dh is inhibited by

phosphate ions.[22] PP-KCl, TRIS and TEA proved to be equally favorable buffers for the demethylation of imipramine. However, with TRIS demethylation values were less constant (Table 2). Pyrophosphate buffer is incompatible with $MgCl_2$ concentrations above 30 μmoles/5 ml incubation mixture. Since the $MgCl_2$ concentration yielding

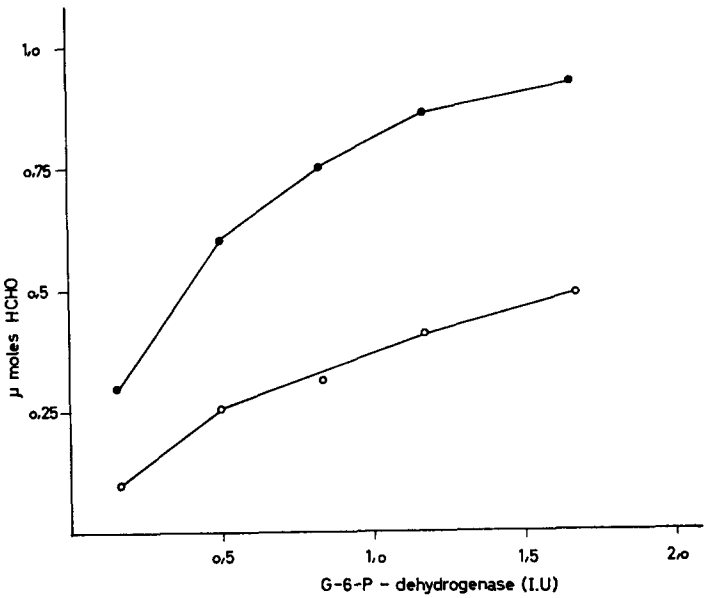


FIG. 1. Demethylation of imipramine (2 μmoles) with various amounts of G-6-P (○—○ 20 μmoles; ●—● 50 μmoles) and G-6-P-dh, other cofactors see Table 2. Five mg microsomal protein. PP-KCl-buffer. Total volume of 5 ml.

TABLE 2. DEMETHYLATION OF IMIPRAMINE IN VARIOUS BUFFER SOLUTIONS*

Buffer solution (pH 7·5, 0·15 M)	% imipramine demethylated	
	5 mg microsomal protein	10 mg microsomal protein
Pyrophosphate/KCl	44 ± 8 (5)	80 ± 12 (5)
TRIS	44 ± 17 (4)	52 ± 21 (3)
Triethanolamine-HCl-EDTA	49 ± 8 (5)	83 ± 7 (6)

* Conditions: Each flask contained 2 μmoles imipramine, microsomal fraction, 50 μmoles G-6-P, 1 μmole NADP, 1·3 I.U. G-6-P-dh. 30 μmoles $MgCl_2$, 100 μmoles niacin, 60 μmoles semicarbazide-HCl. Total volume: 5 ml. In brackets: number of experiments.

optimal demethylation was found to be 90 μmoles/5 ml (Fig. 2), TRIS was replaced by TEA buffer. The increase in substrate (imipramine) concentration above 2 or 3 μmoles per 5 ml incubation mixture resulted in a decrease of the absolute demethylation rate (Fig. 3).

Linear increase in demethylation of imipramine with increasing concentrations of microsomal protein occurs only up to 7–10 mg microsomal protein per 5 ml incubation

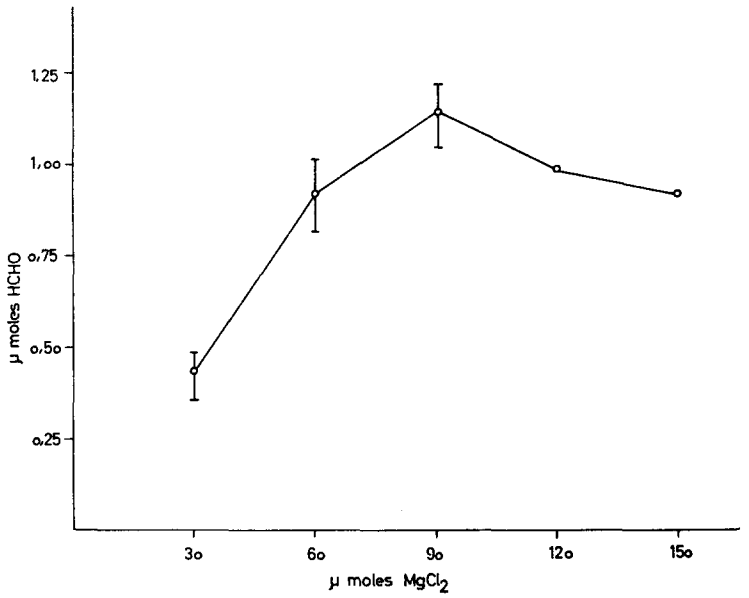


FIG. 2. Demethylation of imipramine (2 µmoles) with various concentrations of $MgCl_2$, other cofactors see Table 2. Five mg microsomal protein, TEA-buffer. Total volume of 5 ml.

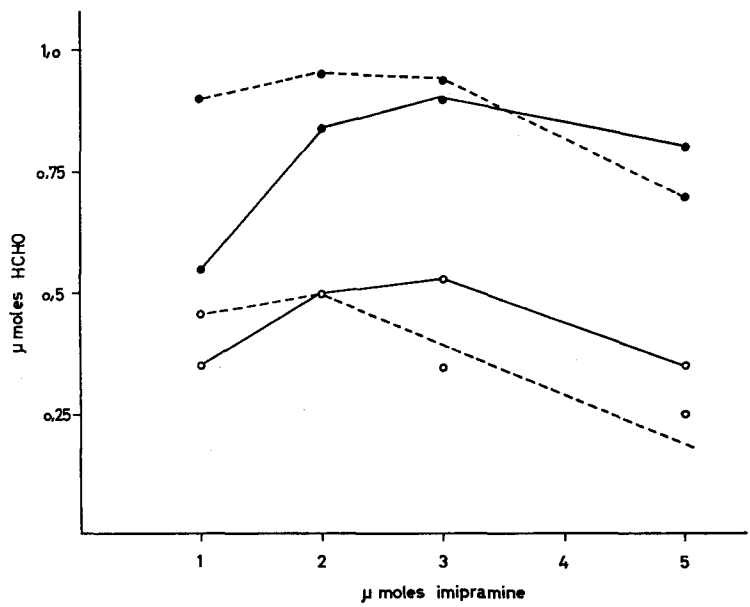


FIG. 3. Effect of imipramine concentration on the rate of demethylation. Cofactors see Table 2. Total volume of 5 ml. ○— 5 mg, ●— 10 mg microsomal protein. (——) TEA-buffer, (– – –) PP-KCl-buffer.

mixture. Above this value the increase is considerably slowed down (Fig. 4). The rate of demethylation is remarkably increased when imipramine is incubated with 9000 *g* supernatant as compared to pure microsomal fraction containing equal amounts of microsomal protein and added cofactors (Fig. 5). These results were used to set up a

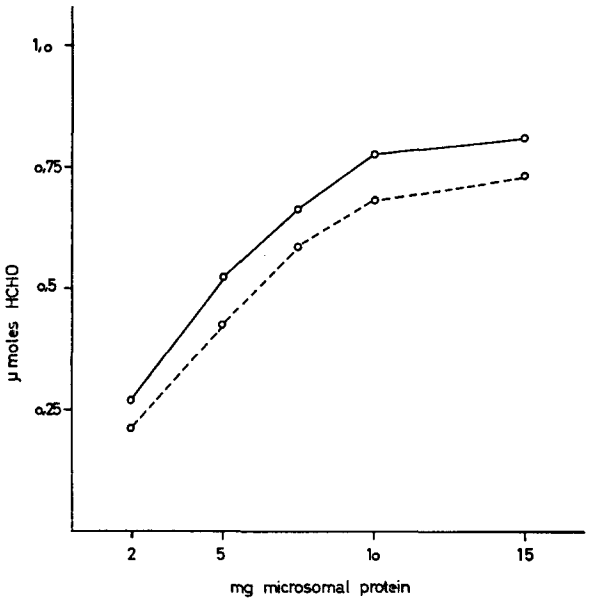


FIG. 4. Demethylation of imipramine (2 µmoles) by various amounts of liver microsomes (in terms of microsomal protein). Cofactors see Table 2. (——) TEA-buffer, (– – –) PP-KCl-buffer. Total volume of 5 ml.

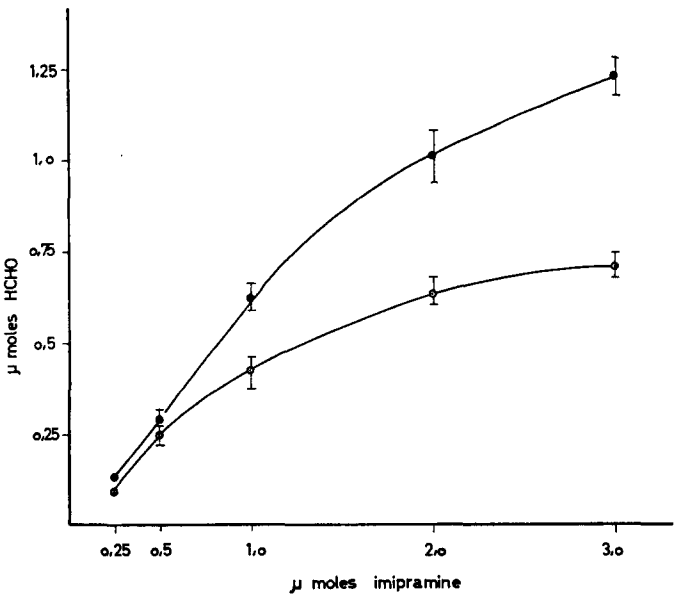


FIG. 5. Demethylation of various concentrations of imipramine by microsomal suspension (○—○) and by 9000 *g* supernatant (●—●). Enzyme corresponding to 250 mg liver. Standard incubation mixture (see Methods).

standard incubation system for optimal metabolic turnover of imipramine. The incubation system is described in Methods and has been used for all the following experiments.

Metabolism of imipramine

2 μmoles imipramine were incubated for 1 hr in the standard incubation system (see Methods). The formaldehyde produced was determined as a measure for the total demethylation. Unchanged imipramine and its metabolites were extracted and separated on thin layer and/or paper chromatography. The sum total recovered was taken as 100 per cent since no imipramine-like material could be detected in the aqueous phase. Besides unchanged imipramine five out of ten known metabolites could be detected after 1 hr incubation time. Two-thirds of the initial imipramine were metabolized; about one half by demethylation. The metabolic pattern proved considerably constant in all experiments. The results are summarized in Table 3.

TABLE 3. THE METABOLITES FORMED *in vitro* BY 2 μMOLES (100%) IMIPRAMINE IN 1 hr

Metabolites	Amount in % ± S.D.
Imipramine (unchanged)	35 ± 5·6
Desmethylimipramine	45 ± 4·7
2-OH-imipramine	10 ± 4·0
Imipramine-N-oxide	5 ± 1·7
2-OH-desmethylimipramine	3 ± 1·5
Iminodibenzyl	2 ± 2·0

* Conditions: Standard incubation mixture (see Methods). Imipramine and metabolites were determined by quantitative chromatography. Mean values from seven experiments.

Chromatography of comparable and higher amounts of the imipramine samples used showed no spots except for imipramine, neither could any formation of metabolites be detected after incubation of imipramine under standard conditions in the absence of liver preparation.

2 μmoles imipramine incubated in the standard system produced 0·82 ± 0·21 μmoles formaldehyde per hr, i.e. 41 per cent total demethylation, thirteen experiments. The corresponding figure drawn out of Table 3 is 45 per cent DMI × 3 per cent OH-DMI = 48 per cent.

Time course of imipramine metabolism

The incubation of imipramine has been run at a 10-fold scale (20 μmoles in 50 ml). In order to follow the time course of the metabolic transformation of the substrate and to check the pattern of metabolites at each of the time points 5 ml samples have been taken out at ¼, ½, 1, 2, 4 and 8 hr and treated like above. The results are shown in Fig. 6. The amounts of metabolites varied between the experiments, however, the pattern of metabolism was always identical and thus can be interpreted in terms of metabolic pathways. In addition to imipramine and its five metabolites detectable after 1 hr the following metabolites appeared after 2 hr incubation time: Desdimethyl-imipramine and a conjugate of 2-hydroxy-desmethylimipramine.

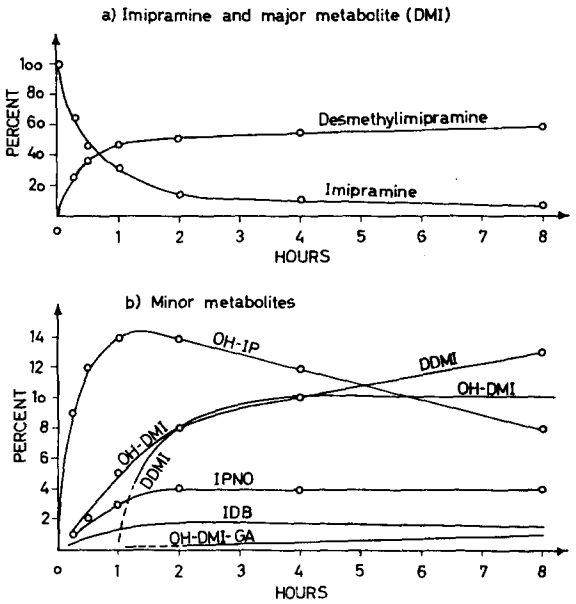


FIG. 6. Time-course of imipramine metabolism. 2 μmoles (100%) imipramine in 5 ml standard incubation mixture. Metabolites and unchanged imipramine assayed chromatographically at different times. Mean values from three experiments corrected for a sum total of 100 per cent.

Metabolism of imipramine metabolites

In order to learn more about the secondary and tertiary metabolites and their rate of formation seven imipramine metabolites available to us were incubated for 1 hr in the same way imipramine had been. The metabolites formed were detected chromatographically (Table 4), and in cases of possible demethylation formaldehyde was

TABLE 4. THE METABOLITES FORMED *in vitro* BY 2 μMOLES (100%) IMIPRAMINE METABOLITES IN 1 hr*

Substrate 100%	Unchanged substrate (per cent)	Metabolites
IP	35	DMI (55%), OH-IP (5%), OH-DMI (2%), IPNO (2%), IDB (1%)
DMI	91	IDB (5%), OH-DMI (2·5%), DDMI (1·5%)
DDMI	>99	IDB (<1%)
IPNO	99	DMI (<1%), IP (<1%), IDB (<1%)
IDB	100	
OH-IP	50	OH-DMI (50%)
OH-DMI	92	OH-IDB (5%), OH-DDMI (3%)
OH-IDB	100	

* Conditions: Standard incubation mixture. Unchanged drugs and metabolites were determined by quantitative chromatography. Mean values from two experiments.

determined (Table 5). 2-hydroxy-imipramine is the only metabolite extensively metabolized by demethylation. Iminodibenzyl and its hydroxy derivative are not metabolized to a measurable amount. The remaining substrates form less than 10 per cent metabolites in 1 hr.

The incubation of the imipramine metabolites shows that in addition to the seven metabolites detectable with imipramine (2–8 hr incubation) two more can be considered as secondary or tertiary metabolites of imipramine, namely 2-hydroxy-desdimethylimipramine and 2-hydroxy-iminodibenzyl. The formation of the conjugate of 2-hydroxy-imipramine could only be detected in the urine of rats treated with large doses of imipramine.[23]

TABLE 5. DEMETHYLATION OF IMIPRAMINE METABOLITES*

Substrate (2 μmoles)	Formaldehyde formed (μmoles)	Demethylation %
Imipramine	1·03	51·5
Desmethylimipramine	0·03	1·5
Imipramine-N-oxide	—	—
2-OH-imipramine	0·99	49·5
2-OH-desmethylimipramine	0·06	3

* Conditions: see Table 4. Demethylation of drug was determined by formaldehyde assay. Mean values from 2 experiments.

Partition coefficients

Partition coefficients of imipramine and its metabolites have been determined by thin layer chromatography of the lipid (chloroform) and aqueous phases (pH 7·5). Among the substrates tested all except 2-hydroxy-desmethyl-imipramine proved extremely liposoluble (Table 6).

TABLE 6. PARTITION COEFFICIENTS OF IMIPRAMINE AND ITS METABOLITES*

Metabolite	% in chloroform
Imipramine	100
Iminodibenzyl	100
2-Hydroxyiminodibenzyl	100
Desmethylimipramine	99·8
Desdimethyl-imipramine	99·6
Imipramine-N-oxide	98·7
2-Hydroxy-imipramine	97·4
2-Hydroxy-desmethyl-imipramine	30

* see Methods.

Non-enzymatic oxidation of imipramine and its metabolites

None of the described incubation experiments transformed imipramine or its metabolites to a measurable amount when the system was lacking enzyme preparation. Neither could the impurities of commercial imipramine—as described by Adank and Hammerschmidt[24]—be detected under the conditions used in our experiments. Only our samples of 2-hydroxy-imipramine and its desmethyl analogue contained measurable amounts of impurities which, however, could be accounted for in the experiments. As an additional means to avoid non-enzymatic artifacts and in order to get information on the stability of imipramine and its metabolites the substances were treated with oxidants. The following types of experiments were carried out: (1) Solutions and evaporation residues were left standing in air, (2) oxidation with hydrogen peroxide

(3 per cent and 30 per cent) and (3) preapplication to thin layer plates and paper and running after a delay of hours or days.

The results from these experiments allow the following conclusions: Oxidation of imipramine results mainly in formation of iminodibenzyl. Under severe conditions traces of imipramine-N-oxide, desmethylimipramine and 2-hydroxy-imipramine are formed. Desmethyl- and desdimethylimipramine are even more resistant to oxidation. The former yields trace amounts of iminodibenzyl, desdimethylimipramine and 2-hydroxy-desmethylimipramine, the latter only iminodibenzyl which is very resistant to further oxidation under the conditions imposed.

DISCUSSION

Drug metabolism studies with microsomal enzyme preparations have become a widespread technique. The composition of incubation mixtures used, however, varies almost from one author to another. We tried to establish optimum conditions for our investigation by testing the influences of experimental conditions on demethylation of imipramine which is the predominant metabolic pathway of this drug. Though these experiments were not the aim of this study their results shall be briefly discussed because they may shed some light on the action and requirements of microsomal drug enzymes on the substrate under investigation.

The finding that demethylation of imipramine is greatly enhanced by increasing concentrations of G-6-P and/or G-6-P-dh suggests that these cofactors, i.e. the amount of $NADPH_2$ formed is limiting the reaction rate rather than the enzyme. Triethanolamine buffer, which apparently has not yet been used in drug metabolism studies *in vitro*, proved to yield high and constant demethylation values, partly due to its compatibility with optimum concentrations of the cofactor, magnesium. Regression of the demethylation rate occurring with imipramine concentrations above 2–3 μmoles per 5 ml—apparently an enzyme inhibition by excess of substrate—reminds one of the finding of Kato *et al.*[25] that imipramine inhibits microsomal drug-metabolizing enzymes. The rate of demethylation of imipramine being proportional to the enzyme concentration only at low concentrations of microsomal protein confirms the findings of Gillette.[26] This author could show that the reason for this is the high binding of imipramine to microsomes,[27] resulting in decreased concentrations of free drug. The enhanced demethylation rate obtained by using 9000 *g* supernatant instead of microsomal suspension (equal amounts of microsomal protein and added cofactors) might be due to the additional cofactor content of cytoplasma fraction. Relative binding of imipramine to cytoplasmic proteins is ten times lower than to microsomes.[27]

A number of authors were able to find a total of ten metabolites of imipramine. This, however, was achieved by using a variety of techniques and several species. Only three of these products (Table 1) have been found to be produced in the rat. We were able to show that all ten metabolites of imipramine are formed in the rat, nine of them *in vitro*. This was done by studying the metabolism of imipramine metabolites with microsmal enzyme preparations and using convenient and highly sensitive thin layer chromatographic methods

There are several methodological limitations to this study. For instance the individual differences in metabolic activity can be considerable even in rats of the same strain. Microsomal preparation in an incubation mixture must be used as no pure

drug metabolizing enzymes are available. The quantitative evaluation methods of paper- and thin-layer chromatograms are rather semi-quantitative. Visual comparison of spot intensities with standard spots proved to be almost as accurate. However, the results obtained showed small deviations, and the qualitative patterns were almost identical. In addition the chromatographic results for demethylation were confirmed by determination of formaldehyde produced. Results of demethylation also are in fairly good accordance with the results obtained by Dingell *et al.*[13, 28] with extraction and photofluorimetry of imipramine and desmethylimipramine.

Imipramine is intensely metabolized quantitatively and qualitatively by rat liver microsomes. 65, 85 and 95 per cent of imipramine are metabolized during 1, 2 and 8 hr respectively under the described experimental conditions. By far the major metabolic pathway of imipramine is N-demethylation to desmethylimipramine which can accumulate due to its considerable biochemical stability. This confirms results obtained *in vitro* and *in vivo* by other authors.[5, 13, 28]

Theoretical considerations as to the sequence of the metabolic steps agree with the comparison of metabolite distribution obtained by incubation of imipramine for various durations (Fig. 7). Thus three metabolites can definitely be regarded as primary, i.e. being formed directly from imipramine. Other metabolites must be considered as secondary ones, i.e. formed from primary metabolites. Together with the results from the separate incubations of the imipramine metabolites the list of the secondary metabolites can be completed and a series of tertiary metabolites emerges. There is, however, no absolute distinction between the three series of metabolites. For instance iminodibenzyl, referred to as secondary metabolite in Fig. 7—because it is more rapidly formed from DMI—is also formed from imipramine directly.

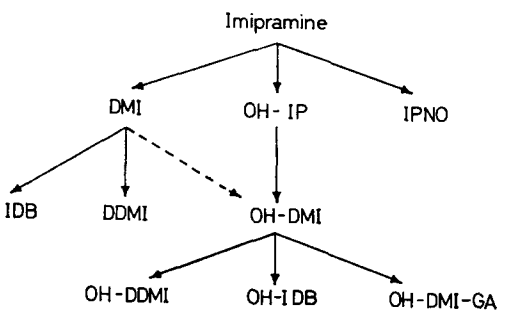


FIG. 7. Metabolism of imipramine. Formation of primary, secondary and tertiary metabolites by rat liver microsomes. Symbols see Table 1.

A total of 16 metabolic pathways relating imipramine with its ten metabolites as well as the metabolites among each other could be detected: 5 N-demethylations, 2 aromatic hydroxylations, 5 sidechain N-dealkylations (aminopropyl), 1 N-oxidation, 1 N-oxide-reduction and 2 conjugations.

Though the demethylation rate of imipramine is very high the further demethylation of DMI to DDMI proceeds very slowly, the ratio being 50: 1. The same case exists with the analog series amitriptyline—nortriptyline—desmethylnortriptyline.[29, 30] Two different demethylases rather than potent blockade by DMI

formed are likely because the demethylation of imipramine proceeds also in the presence of high amounts of DMI. The equal demethylation rate of OH-imipramine to OH-DMI is suggesting that one enzyme is demethylating both substrates. Again the demethylation of OH-DMI to OH-DDMI is almost negligible. The next important pathway is the 2-hydroxylation of imipramine to OH-IP. Its rate is about 1/5 that of demethylation. The corresponding hydroxylation of DMI proceeds at a slower rate and DDMI is not hydroxylated to a measurable amount. OH-IDB is not formed by hydroxylation of IDB but by chain dealkylation of OH-DMI. The same reaction type converts imipramine, DMI, DDMI and IPNO to IDB, however, only at small rates. Imipramine is oxidized to imipramine-N-oxide, but very limited amounts are formed. Still smaller is the rate of N-oxide being reduced to imipramine or demethylated to DMI. The OH-derivatives of imipramine and DMI are conjugated to glucuronides or/and sulfates. Herrmann *et al.*[2, 3] describe the conjugates as glucuronides. The conjugation rates of these hydroxy-compounds in the rat are remarkably low. A scheme of all the detected metabolic pathways is given in Fig. 8.

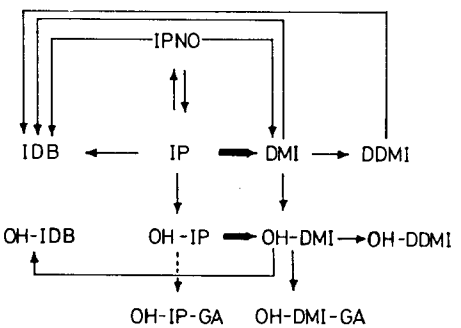


FIG. 8. Metabolic pathways of imipramine and its metabolites in rat liver microsomes. Symbols see Table 1.

major pathways.
minor pathways.
in vivo with high doses of imipramine.

Of interest in respect to the mechanism of microsomal N-demethylation is the question as to whether N-oxides are intermediary compounds between tertiary and secondary amines.[31] In our time-course studies of the metabolism of imipramine IPNO is steadily increasing during the first 2 hr, i.e. even when the increase in DMI is considerably reduced and the supply of imipramine low. This would not be expected if IPNO were an intermediary product rapidly converted to DMI. Furthermore IPNO on incubation with the microsomal enzyme preparation produces extremely little DMI and hardly any other metabolites. The theoretically increased polarity of IPNO as compared to imipramine or DMI might suggest that IPNO does not reach the microsomal enzymes presumably surrounded by a lipoid barrier.[32, 33] However, the chloroform–water partition coefficients (Table 6) show that IPNO is still very liposoluble, even more so than OH-IP which is rapidly demethylated by microsomal enzymes. These results suggest that IPNO is no intermediary product in N-demethylation, so that the presence of both a rapidly acting demethylating system and a far

less active N-oxidase must be assumed. Similar findings have been reported by McMahon and Sullivan[34] studying the demethylation of 1-propoxyphene.

Similarly the high liposolubilities of IDB, OH-IDB, DMI and DDMI suggest that their extreme or relative stability in contact with microsomal enzymes of rat liver is conclusive.

As shown in Fig. 8 analogue metabolites are not always converted by identical reactions or at least not at the same rate. For instance imipramine and DMI but not IDB and DDMI are 2-hydroxylated, imipramine and OH-IP but very little DMI and OH-DMI are demethylated, imipramine and OH-DMI but not OH-IP are side chain dealkylated, OH-DMI but not OH-IP is conjugated *in vitro*. This suggests a relatively high degree of substrate specificity of one enzyme type and therefore various types of demethylases, hydroxylases, glucuronide transferases have to be assumed. By computing the results of the time-course studies and the sequence of metabolite formation the following approximate values for relative enzyme activities of the rat liver microsomal enzyme system were obtained (% conversion per 0·1 hr.):

Disappearance of imipramine	10
IP ⟶ DMI	9
OH-IP ⟶ OH-DMI	8
IP ⟶ OH-IP	1·3
Disappearance of DMI	1

These values reproduce the theoretical amounts of primary metabolites formed—and when taking into account the formation of secondary metabolites—meet the experimental curves of metabolite formation during the first hour of imipramine metabolism. For instance "imipramine demethylase" (relative activity = 9) would account for more DMI formed than found experimentically, however, part of the DMI is constantly transformed into IDB, DDMI and OH-DMI (relative activity = 1). Similarly imipramine is relatively slowly hydroxylated (relative activity = 1·3), but OH-IP is rapidly demethylated to OH-DMI (relative activity = 8). Thus the level of the primary metabolite, OH-IP, declines after a peak at 1 hr and is surpassed by the level of the secondary metabolite, OH-DMI, after about 6 hr.

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REFERENCES

1. W. SCHINDLER, *Helv. Chim. Acta* **43**, 35 (1960).
2. B. HERRMANN and R. PULVER, *Archs Int. Pharmacodyn.* **126**, 454 (1960).
3. B. HERRMANN, W. SCHINDLER and R. PULVER, *Medna. exp.* **1**, 381 (1960).
4. F. SULSER, J. WATTS and B. B. BRODIE, *Ann. N.Y. Acad. Sci.* **96**, 279 (1962).
5. J. R. GILLETTE, J. V. DINGELL, F. SULSER, R. KUNTZMAN and B. B. BRODIE, *Experientia, Basel* **17**, 417 (1961).
6. K. BERNHARD and H. BEER, *Helv. physiol. pharmac. Acta* **20**, 114 (1962).
7. B. B. BRODIE, M. H. BICKEL and F. SULSER, *Medna. exp.* **5**, 454 (1961).
8. F. SULSER, M. H. BICKEL and B. B. BRODIE, in *Pharmacological Analysis of Central Nervous Action* (Ed. W. D. M. PATON), vol. 8, p. 123/Pergamon Press, Oxford (1962).
9. V. FISHMAN and H. GOLDENBERG, *Proc. Soc. exp. Biol. Med.* **110**, 187 (1962).
10. J. IM OBERSTEG and J. BÄUMLER, *Arch. Tox.* **19**, 339 (1962)

11. B. HERRMANN, *Helv. physiol. pharmac. Acta* **21**, 402 (1963).
12. J. V. DINGELL, F. SULSER and J. R. GILLETTE, *Fedn. Proc.* **21**, 184 (1962).
13. J. V. DINGELL, F. SULSER and J. R. GILLETTE, *J. Pharmac. exp. Ther.* **143**, 14 (1964).
14. G. BEISENHERZ, H. J. BOLTZE, T. BÜCHER, R. CZOK, K. H. GARBADE, E. MEYER-ARENDT and G. PFEIDELER, *Z. Naturforsch.* **80**, 555 (1953).
15. T. NASH, *Biochem. J.* **55**, 416 (1953).
16. J. COCHIN and J. AXELROD, *J. Pharmac. exp. Ther.* **125**, 105 (1959).
17. J. V. DINGELL, personal communication.
18. J. R. PELKA and L. D. METCALFE, *Anal. Chem.* **37**, 603 (1965).
19. J. R. GILLETTE, W. GRIEB and H. SASAME, *Fedn. Proc.* **22**, 366 (1963).
20. G. GOMORI, *Proc. Soc. exp. Biol. Med.* **62**, 33 (1946).
21. G. GOMORI, *Proc. Soc. exp. Biol. Med.* **68**, 354 (1948).
22. H. THEORELL, *Biochem. Z.* **275**, 416 (1935).
23. M. H. BICKEL and M. BAGGIOLINI, in preparation.
24. K. ADANK and W. HAMMERSCHMIDT, *Chimia* **18**, 361 (1964).
25. R. KATO, E. CHIESARA and P. VASSANELLI, *Biochem. Pharmac.* **12**, 357 (1963).
26. J. R. GILLETTE, in *Drugs and Enzymes* (Eds. B. B. BRODIE and J. R. GILLETTE), p. 9 Pergamon Press, Oxford (1965).
27. J. V. DINGELL, W. A. M. DUNCAN and J. R. GILLETTE, *Fedn. Proc.* **20**, 173 (1961).
28. J. V. DINGELL, PhD.-thesis, Georgetown University, Washington, D.C. (1962).
29. H. B. HUCKER, *Pharmacologist* **4**, 171 (1962).
30. R. E. MCMAHON, F. J. MARSHALL, H. W. CULP and W. M. MILLER, *Biochem. Pharmac.* **12**, 1207 (1963).
31. M. S. FISH, C. C. SWEELEY, N. M. JOHNSON, E. P. LAWRENCE and E. C. HORNING, *Biochem. Biophys. Acta* **21**, 196 (1956).
32. L. E. GAUDETTE and B. B. BRODIE, *Biochem. Pharmac.* **2**, 89 (1959).
33. R. E. MCMAHON, *J. Med. Pharm. Chem.* **4**, 67 (1961).
34. R. E. MCMAHON and R. R. SULLIVAN, *Life Sci.* **3**, 1167 (1964).