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IRREVERSIBLE PROTEIN BINDING OF [^{14}C]IMIPRAMINE WITH RAT AND HUMAN LIVER MICROSOMES*

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Abstract—After incubation of [^{14}C]imipramine with rat liver microsomes up to 0·7 nmole/mg was irreversibly bound per mg of microsomal protein. If albumin was added to the microsomal incubations [^{14}C]imiprämine was also irreversibly bound to this protein. The irreversible binding of imipramine to protein was determined by exhaustive solvent extraction and charcoal adsorption, and measurement of the remaining ^{14}C-radioactivity in the protein. The binding reaction was dependent on oxygen, NADPH, microsomal protein content and substrate concentration. It was inhibited by CO and SKF 525-A. Pretreatment of rats with phenobarbital did not increase the amount of imipramine irreversibly bound to protein. Glutathione and other cysteine derivatives diminished the binding, whereas incubation with the epoxide hydrase inhibitor trichloropropene oxide resulted in an increase of imipramine irreversibly bound to protein. The results favour the concept that irreversible protein binding of imipramine is catalyzed by a cytochrome P-450-dependent hydroxylation via an epoxidation step. Irreversible protein binding of imipramine was also detectable with three samples of human liver microsomes.

The metabolism of imipramine, a widely used antidepressive agent has been extensively studied *in vitro* and *in vivo* in several species [1–5].

Liver injuries, such as intrahepatic cholestasis [6] and hepatic necrosis [7], as well as thrombocytopenia and agranulocytosis [8–10], various skin reactions [11] and other allergic reactions [12] have been reported after medication with imipramine.

A correlation between metabolism of imipramine and the side effects has been discussed, but never established. Adverse effects of drugs are probably mediated by an immunochemical mechanism [13–16], but no conclusive evidence has been presented. Because of the inherent difficulties in demonstrating specific antibodies which could prove an immunological process we tried first to elucidate, whether imipramine is able to give rise to a compound with antigenic properties.

Antibodies are produced against a drug with low molecular weight only if a chemical substance is bound covalently to a macromolecular carrier, e.g. a protein [15–17]. The biochemical mechanism of this reaction has been established for only a few drugs [16–23].

In previous studies we examined the mechanism of irreversible binding of ethynylestradiol to proteins [24, 25] and nucleic acids [25]. After having demonstrated the catalytic action of drug metabolizing enzymes in liver microsomes as being an essential requirement for the formation of metabolites which can bind covalently to macromolecules [24, 25], we wondered whether a similar mechanism might be responsible for an irreversible protein binding of tricyclic psychopharmaca, especially of imipramine, and what are the conditions which cause such a binding.

In a preliminary report Uehleke [42] supposed that imipramine is covalently bound to protein of rabbit liver microsomes. We demonstrated, with *in vitro* experiments, an irreversible binding of imipramine to rat liver microsomes and albumin. We examined also samples of human liver microsomes in order to find out whether they behave similarly.

MATERIALS AND METHODS

Materials. [^{14}C]Imipramine [*N*-(3-dimethylaminopropyl)imino-dibenzyl (methylene-^{14}C)hydrochloride] was obtained from Radiochemical Center, Amersham. The specific radioactivity was 4·5 mCi/m-mole. Non-radioactive imipramine was kindly donated by Ciba-Geigy AG, Basel, Switzerland. The chemical purity of both compounds was higher than 99 per cent.

Bovine serum albumin was obtained from Behringwerke, Marburg, L-glutathione and other biochemicals from Boehringer, Mannheim, L-cysteine and all standard laboratory chemicals from Merck, Darmstadt, L-cysteine-methylester from Fluka, Buchs, Switzerland, *N*-acetyl-L-cysteine from Schuchhardt, München, and SKF 525-A (2-diethylaminoethyl-2,2-diphenylvalerate.HCl) from Smith, Kline & French, Philadelphia. 1,1,1-Trichloropropene oxide was a generous gift from Dr. F. Oesch, Biocenter Basel, Switzerland.

Animals. Male Wistar rats weighing 220–280 g were used. In most experiments the rats were pretreated with phenobarbital to induce the microsomal mixed-function oxidase system. These rats received a single intraperitoneal injection of 80 mg/kg followed by 0·1% phenobarbital in the drinking water for 5 days. The rats were starved for 1 day before being killed.

* The results were presented at the Pharmacology Meeting Graz, 1974 (H. Kappus, H. M. Bolt and H. Remmer; *Naunyn Schmiedeberg's Arch. Pharmac.* Suppl. **285**, R 38, 1974).

Microsomal incubations. Rat and human liver microsomes were prepared and incubations were performed as described previously [26].

Microsomes from each rat were prepared separately. Microsomal incubations were preformed in 1·5-ml Eppendorf reaction tubes with an NADPH regenerating system. The total incubation volume was 1 ml. The reaction mixture contained 5 mM $MgCl_2$, 8 mM D,L-Na-isocitrate, 1 mM $NADP^+$, 0·116 mM [^{14}C]imipramine (sp. act. 0·62 mCi/m-mole) and 5 μl/ml isocitrate dehydrogenase (from pig heart, Boehringer) in 0·25 M Tris–HCl buffer pH 7·5. Microsomal protein content was 0·5–1·5 mg/ml, determined according to Lowry *et al.* [27].

After 5 min of preincubation, the microsomal enzyme reaction was started by addition of $NADP^+$ and the tubes were shaken at 37° under air for 90 min.

In experiments determining the dependence on microsomal protein concentration, incubations were performed with different protein concentrations of pooled microsomes from four male rats treated with phenobarbital.

In the time dependence experiments, total incubation volume was increased to 5 ml and 0·5 ml was withdrawn at intervals and added to 1 ml of ethanol.

Liver microsomes were obtained from three patients. Two of them were pretreated with rifampicin for 6 days in order to measure the induction of drug metabolism as reported elsewhere (for details see Ref. 28). The third patient received no rifampicin. Incubations with these microsomes were performed with the same medium as rat liver microsomes, but had a lower protein content.

Extraction procedure. The extraction procedures were carried out in 1·5 ml Eppendorf reaction tubes. 0·5 ml of the incubation mixture was added to 1 ml ethanol to precipitate the microsomal protein and to remove the lipids. Trichloroacetic acid was not used in order to prevent splitting of acid labile protein binding. The precipitate was centrifuged, the supernatant discarded and 0·5 ml 70% ethanol was added to the protein pellet. The precipitate was resuspended in 70% ethanol, using a teflon pestle adapted to the Eppendorf tube. The protein was centrifuged again and extracted using 0·5 ml of different solvents in the same manner. The following extraction sequence was used: twice with 0·25 M Tris–HCl buffer (pH 7·5), once with 70% ethanol, once with 96% ethanol at 25°, once with 96% boiling ethanol, twice with acetone–chloroform (4:1) and finally once with 70% ethanol.

All extraction supernatants were tested for radioactivity. After the last extraction no further radioactivity could be removed from the protein, even if other solvents were used. The radioactivity bound to the microsomal protein remained constant after these extraction procedures. Therefore, this extraction method removes the original imipramine as well as all metabolites reversibly bound to protein. Protein loss was very low during the procedure.

After extraction, the microsomal protein was dissolved in Hyamin® hydroxide, the contents of the tube were transferred to a counting vial and radioactivity was measured in 10 ml Bray's solution [29]. Values were corrected for quenching by internal or external standardization. From the specific radioactivity and the protein content in the incubation mixture, nmoles of imipramine metabolites irreversibly bound per mg microsomal protein were calculated.

Charcoal adsorption. Bovine serum albumin (10 mg/ml) was added to the incubation medium in several experiments in order to determine whether imipramine is also irreversibly bound to an added soluble protein. After incubation the tubes were cooled, the microsomes centrifuged, and microsomal protein denatured with 70% ethanol and the extraction procedure performed as described above. The supernatant containing the albumin was shaken three times with an equal volume of charcoal suspension (1% norit A and 0·01% dextran in 0·25 M Tris–HCl buffer, pH 7·5) as described previously in our experiments of irreversible binding of estrogens [24, 25]. Protein was then dissolved in Hyamin® hydroxide and radioactivity determined in 10 ml Bray's solution [29].

Inhibition experiments. (a) Inhibitors of cytochrome P-450: experiments were carried out in the presence of CO (95% CO and 5% O_2) or SKF 525-A (1 mM), specific inhibitors of the microsomal mixed-function oxidase cytochrome P-450. (b) Trapping with SH-compounds: the concentration of sulfhydryl compounds in the incubation was 1 mM, that of albumin 10 mg/ml. (c) Inhibition of epoxide hydrase: Trichloropropene oxide (10 mM), an uncompetitive inhibitor of epoxide hydrase, was used to inhibit the transformation of a possibly formed epoxide of imipramine to its dihydrodiol-derivative.

RESULTS

Incubation of [^{14}C]imipramine with rat liver microsomes leads to an irreversible binding of 1–2 per cent of the radioactivity to microsomal protein after exhaustive extraction using various solvents.

This small amount of bound [^{14}C]imipramine, can be detected only if it is determined directly in the protein pellet; such a low binding cannot be calculated from recovery studies.

Figure 1 shows that the irreversible protein binding of imipramine or of one of its metabolites depends

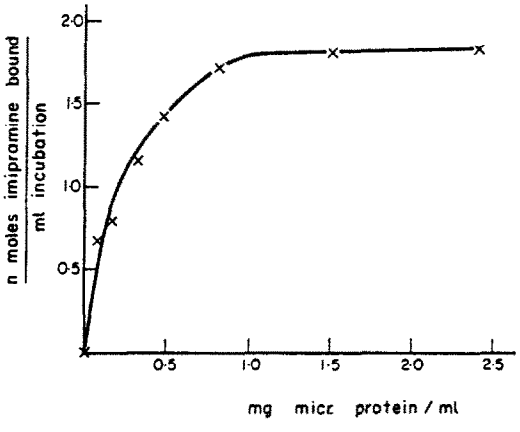

Fig. 1. Irreversible protein binding of [^{14}C]imipramine (0·116 mM) catalyzed by pooled liver microsomes from four phenobarbital pretreated rats in the presence of a NADPH regenerating system after 90 min incubation. Dependence on the microsomal protein concentration (one experiment). Total binding is shown.

Table 1. Irreversible protein binding of [^{14}C]imipramine (0·116 mM) catalyzed by rat liver microsomes in presence of NADPH regenerating system after 90 min incubation

	Imipramine metabolites irreversibly bound to 1 mg microsomal protein (nmoles)	
	With phenobarbital pretreament	Without pretreatment
Without NADP$^+$	0·02 ± 0·003 (n = 10)	0·02 ± 0·005 (n = 4)
With NADP$^+$	0·70 ± 0·20* (n = 18)	0·53 ± 0·13* (n = 4)

With boiled microsomes no [^{14}C]imipramine was bound to protein.

* Difference is statistically not significant (0·2 > P > 0·1 group *t*-test).

on the microsomal protein content in the incubation. Saturation is reached with 1 mg microsomal protein/ml. The saturation curve signifying the irreversible binding of imipramine to protein is very similar to the curve which demonstrates irreversible binding of ethynylestradiol to microsomal protein as described previously [24]. Marks and Hecker [30] for estradiol, Potter *et al.* [22] for acetaminophen and Uehleke *et al.* [20] for halothane found an identical dependence of covalent protein binding on the microsomal protein content. Therefore irreversible protein binding of xenobiotics and also of imipramine, is limited by the microsomal protein concentration as is well known for microsomal hydroxylation reactions [31].

No imipramine metabolite was irreversibly bound to microsomal protein if microsomes were boiled before the incubation (Table 1), indicating that a microsomal enzyme step is involved.

Binding of imipramine radioactivity to microsomal protein is a time dependent process (Fig. 2), which is linear between 5 and 45 min and is almost complete after 90 min. This might be regarded as a further indication for an enzyme reaction. We used an incubation time of 90 min if not otherwise stated.

The irreversible protein binding of imipramine metabolites is also dependent on the substrate concentration (Fig. 3). The imipramine concentration

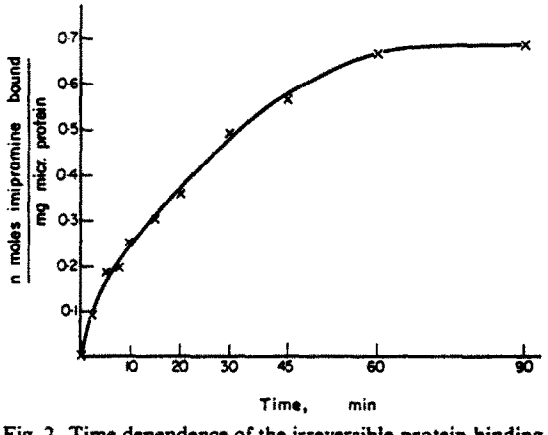

Fig. 2. Time dependence of the irreversible protein binding of [^{14}C]imipramine (0·116 mM) catalyzed by liver microsomes from phenobarbital pretreated rats in the presence of a NADPH regenerating system. Mean values of two experiments.

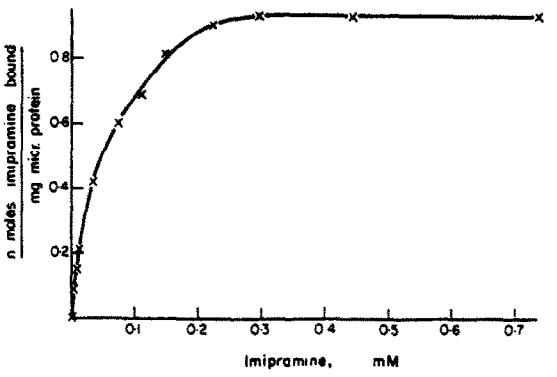

Fig. 3. Irreversible protein binding of [^{14}C]imipramine catalyzed by liver microsomes from phenobarbital pretreated rats in the presence of a NADPH regenerating system after 90 min incubation. Dependence on substrate concentration. Mean values of two experiments.

chosen for standard experiments (0·116 mM) was close to saturation conditions.

Figure 4 demonstrates that the irreversible binding of imipramine to microsomal protein is directly proportional to the concentration of NADPH which was added to the incubation instead of the NADPH regenerating system. Concordantly, only an insignificant portion of imipramine radioactivity is bound to the protein if NADP$^+$, the precursor of NADPH in our regenerating system, is omitted (Table 1). Under these conditions the radioactivity bound to protein amounted only to 3 per cent (0·02 nmole) of the total imipramine bound (0·70 nmole) under optimal conditions (Table 1). NADPH-dependency is further evidence for an enzyme reaction being involved in the irreversible binding of imipramine. The protein binding of imipramine was not significantly increased in microsomes from phenobarbital treated rats (Table 1).

Two specific inhibitors of the microsomal mixed-function oxidase–cytochrome P-450 system, CO and SKF 525-A, significantly diminished the binding reaction of imipramine to microsomal protein (Table 2).

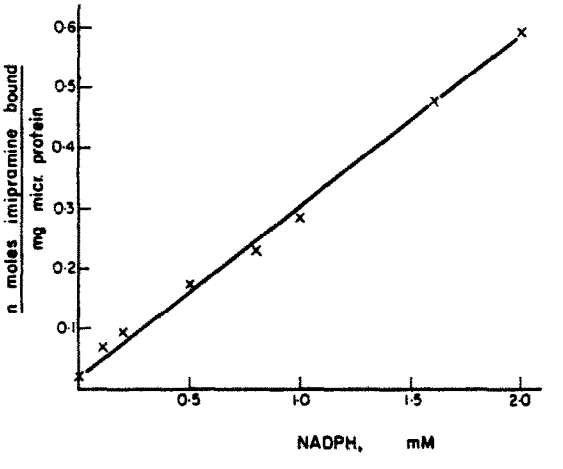

Fig. 4. NADPH-dependence of the irreversible protein binding of [^{14}C]imipramine (0·116 mM) catalyzed by liver microsomes from phenobarbital pretreated rats in the presence of different NADPH concentrations after 90 min incubation. NADPH was added instead of the NADPH regenerating system (one experiment).

Table 2. Irreversible protein binding of [^{14}C]imipramine (0·116 mM) catalyzed by liver microsomes of phenobarbital pretreated rats in presence of NADPH regenerating system after 90 min incubation. Effect of CO and SKF 525 A (1 mM), different SH-compounds (1 mM) and trichloropropene oxide (10 mM)

Imipramine metabolites irreversibly bound to 1 mg microsomal protein (nmoles)		Per cent inhibition
Control (see Table 1)	0·70 ± 0·20* (n = 18)	—
With 95% CO 5% O_2	0·08 ± 0·02 (n = 4)	88·6
With SKF 525 A	0·19 ± 0·01 (n = 4)	72·8
With L-glutathione	0·13 ± 0·03 (n = 14)	81·4
With L-cysteine-Methylester	0·08 ± 0·02 (n = 4)	88·6
With L-cysteine	0·12 ± 0·02 (n = 4)	82·8
With *N*-acety-L-cysteine	0·43 ± 0·13 (n = 4)	38·5
With bovine serum albumin (10 mg/ml)	0·48 ± 0·11 (n = 8)	31·5
With trichloropropene oxide	1·07 ± 0·11* (n = 4)	—

All differences are statistically significant.
* 0·05 > P > 0·01 (paired comparison).
For all other differences P < 0·001 (paired comparison).
Under nitrogen (without oxygen) no [^{14}C]imipramine was bound to the protein.

On the other hand, no irreversible binding of imipramine to protein occurred if oxygen was substituted by nitrogen.

The dependence of the binding of imipramine to microsomal protein on a microsomal enzyme, on substrate concentration, oxygen and NADPH as well as the inhibition of this reaction with CO and SKF 525-A indicates that the binding of imipramine is catalyzed by a mixed-function oxidation step, probably by the unspecific drug metabolizing system involving cytochrome P-450 of liver microsomes.

In order to find out, what kind of metabolite reacts with proteins, we studied the effect of compounds known to trap reactive metabolites formed in microsomes. All the SH-substances tested decreased the irreversible protein binding of imipramine (Table 2). Cysteine, cysteine-methylester and glutathione almost totally blocked this binding. The inhibition by *N*-acetyl-cysteine and albumin was less pronounced. These substances might remove the reactive imipramine metabolite formed by microsomal oxidation, probably by establishing a chemically stable thioether bond.

If bovine serum albumin was added to microsomal incubation system [^{14}C]imipramine was irreversibly bound to this protein also. No binding was detected without the NADPH regenerating system (Table 3). This binding could also be inhibited by glutathione in accordance with the experiments measuring only microsomal protein. Thus, the binding of an imipramine metabolite to albumin itself may be the reason for the fact that albumin decreases its binding to microsomal protein (Table 2).

Table 3. Irreversible binding of [^{14}C]imipramine (0·116 mM) to bovine serum albumin (10 mg/ml) added to the microsomal incubation, as catalyzed by liver microsomes of phenobarbital pretreated rats in presence of NADPH regenerating system after 90 min incubation

Imipramine metabolites bound to 10 mg albumin by 1 mg microsomal protein (nmoles)	
Without inhibitor	With glutathione (1 mM)
0·51 ± 0·09 (n = 8)	0·10 ± 0·02 (n = 4)

Difference is statistically significant (P < 0·001).
Without oxygen and without NADPH regenerating system, no [^{14}C]radioactivity was bound to albumin. Under an atmosphere of 95% CO/5% O_2 the binding of imipramine to albumin was inhibited to 85 per cent.

The reactive imipramine intermediate may be an epoxide of imipramine or of one of its metabolites. Table 2 shows the results of incubation experiments in the presence of imipramine and trichloropropene oxide, an uncompetitive inhibitor of epoxide hydrase which transforms epoxides to dihydrodiols [32–35]. The inhibition of this enzyme leads to an elevation of irreversible protein binding of imipramine. Therefore, an imipramine epoxide seems to be the reactive metabolite which binds to protein.

Irreversible protein binding of imipramine to human liver microsomes was also measurable (Table 4). The binding depends on NADPH and is inhibited by glutathione and albumin. Correspondingly, imipramine is also bound to albumin itself if added to the incubation mixture. The values should not be quantitatively compared with rat liver microsomal experiments, because the dependence of the irreversible protein binding of imipramine on microsomal protein concentration is not known for human liver microsomes.

The results demonstrate that rifampicin pretreatment induces irreversible protein binding of imipramine, as determined for hydroxylations of drugs, such as *p*-nitroanisole and ethynylestradiol, carried out with identical microsomal suspensions [28].

Table 4. Irreversible protein binding of [^{14}C]imipramine (0·116 mM) catalyzed by human liver microsomes in presence of NADPH regenerating system after 90 min incubation

	Imipramine metabolites irreversibly bound to 1 mg microsomal protein (nmoles)		
	Without inhibitor	With glutathione (1 mM)	With bovine serum albumin (10 mg/ml)
Patient A (rifampicin pretreated)	0·55	0·26	0·34
Patient B (rifampicin pretreated)	0·63	0·21	0·41
Patient C (without pretreatment)	0·13	0·03	N.D.

Without NADPH regenerating system no [^{14}C]imipramine was bound to protein. To albumin itself, imipramine metabolites were also irreversibly bound.

DISCUSSION

The results show that some [^{14}C]imipramine or a metabolite is firmly bound to protein if incubated with liver microsomes in the presence of oxygen and NADPH. The binding seems to be covalent but because the nature of this protein binding is not yet established, we prefer the term "irreversible binding" as used previously in our experiments with ethynylestradiol [24, 25].

The irreversible protein binding of imipramine has to be distinguished from the reversible binding of imipramine to subcellular structures, possibly to proteins, as described by Bickel and Steele [36, 37]. In their experiments they excluded enzyme reactions with imipramine. Therefore, they could not measure irreversible protein binding of imipramine in addition to the extremely high reversible binding of imipramine to microsomes.

The reactive metabolite which is possibly formed by the action of the mixed-function oxidase–cytochrome P-450 system is trapped with SH-compounds (Table 2). To our knowledge, a conjugate with one of these substances, e.g. a glutathione conjugate or a mercapturic acid of imipramine has never been isolated for imipramine *in vitro* or *in vivo*. However, such a metabolite may be quantitatively of minor importance in metabolism studies and might be overlooked.

The *N*-oxidation reaction of the dimethylamino group of imipramine cannot be the prerequisite for its covalent protein binding, because *N*-oxidations are not inhibited by CO and SKF 525-A [3, 38] as is the case for the irreversible protein binding of imipramine (Table 2).

The cytochrome P-450 catalyzed demethylation of one methyl group of imipramine leads to desipramine and further demethylation to the amine, desdimethylimipramine [1–5]. Neither compound should react with sulfhydryl substances.

On the other hand, 2-hydroxylation of imipramine and of its demethylated products is one of the main metabolic steps performed by the cytochrome P-450 system of liver microsomes [1–5, 39, 40]. It is generally assumed that in aromatic hydroxylations and also in 2-hydroxylation of imipramine, an expoxidation step is involved [33]. According to the stability of the epoxide formed, it is converted to the corresponding phenol or further metabolized to a dihydrodiol compound by epoxide hydrase [32–34]. The dihydrodiols are chemically inert [33].

Thioethers arise from the reaction of epoxides with SH-compounds, especially glutathione. Normally thioethers are formed enzymatically by glutathione epoxide transferases, but non-enzymatic conjugations of epoxides with glutathione have also been observed [33]. The elevated irreversible protein binding of imipramine in the presence of an epoxide hydrase inhibitor (Table 2) is evidence for the formation of an epoxide intermediate of imipramine during the binding reaction. In our experiments with epoxide hydrase inhibitor the irreversible protein binding of imipramine might not increase to a higher extent, because trichloropropene-oxide possibly reacts with SH-groups of proteins itself.

The unexpected ineffectiveness of phenobarbital pretreatment to increase the irreversible binding is another indication that epoxide hydrase is involved. Microsomes from phenobarbital pretreated rats catalyze a higher binding of ethynylestradiol to proteins [24]. However, an epoxide is not involved in the formation of the reactive metabolite. As Oesch has shown [35], phenobarbital induces the epoxide hydrase. This might be the reason that an increase of imipramine binding to protein could not be observed in our experiments, if microsomes of phenobarbital treated rats were used. The induction of the hydrase might counteract the increase of the epoxide formation.

The finding, that imipramine metabolites were irreversibly bound to a soluble protein like albumin added to the microsomal incubation, led us to assume that the epoxide, if formed, is stable enough to leave the microsomal membrane in order to react with this protein.

Binding of a drug metabolite to a soluble protein catalyzed by microsomal enzymes should be viewed as an *in vivo* model. Proteins outside the endoplasmic reticulum might combine with the chemically reactive drug metabolite. The altered protein may leave the liver cell and induce the production of antibodies against the drug, which now has a haptenic property. For example imipramine may develop antigenic activity when bound covalently to a plasma protein newly synthesized in the endoplasmic reticulum of the liver.

Microsomes from human liver catalyzed the same reactions, leading to an irreversible binding of imipramine to protein. An antibody against desipramine was described by Rachmilewitz *et al.* [41] in a patient receiving imipramine.

Some adverse effects of imipramine in patients might be due to the formation of antibodies against this drug. Therefore, the demonstration that an imipramine metabolite is irreversibly bound to proteins, catalyzed by a liver enzyme system, is an essential prerequisite. The activity of this enzyme system, dependent on cytochrome P-450, may be responsible for causing side effects of imipramine. However, some other factors like glutathione levels in the cell and epoxide hydrase activity in the endoplasmic reticulum should diminish the extent of the protein binding of imipramine. However, adverse effects might depend more on the tolerance of the immunological system than on the amount of imipramine metabolites bound. Typical cell necrosis in the liver during imipramine therapy was observed by Powell *et al.* [7], but liver injury due to hepatitis or cholestasis has been described more frequently.

The amount of imipramine metabolites bound to protein is very low *in vitro* as well *in vivo* (unpublished observation). Whether the altered protein molecules are sufficient to induce immunological reactions needs to be investigated.

If the immunochemical mechanism of adverse effects against imipramine is caused by its covalent binding to proteins in liver endoplasmic reticulum, our results might be of importance in view of other tricyclic psychopharmaca, which often produce higher incidence of allergic reactions than imipramine.

Studies of other tricyclic psychopharmaca deserve further investigation.

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