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α-NAPHTHYLISOTHIOCYANATE (ANIT) HEPATOTOXICITY AND IRREVERSIBLE BINDING TO RAT LIVER MICROSOMES*

A. MONAEM EL-HAWARI† and GABRIEL L. PLAA‡
Département de Pharmacologie, Faculté de Médecine, Université de Montréal, Montréal, Québec, Canada

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Abstract—The hyperbilirubinemic effect produced by α-naphthylisothiocyanate (ANIT) in rats was significantly altered by different inducers and inhibitors of drug metabolism and protein synthesis, which supports the hypothesis that biotransformation of ANIT to a toxic metabolite is crucial to its cholestatic effect. The binding of a chemically reactive ANIT-derived product to rat liver microsomes was examined *in vitro*. When [^{3}H]ANIT or [^{14}C]ANIT was incubated with rat liver microsomes, radioactivity was bound irreversibly to proteins. This binding occurred in the absence (non-enzymic) and, to greater extent, in the presence (enzymic) of NADPH and oxygen. Enzymic binding was inhibited by carbon monoxide, β-diethylaminoethyl diphenylpropylacetate (SKF 525-A) and piperonyl butoxide, indicating that a cytochrome P-450-dependent, mixed-function oxidase mediated the binding. The enzymic binding was reduced by incubation with glutathione and other thiol-containing compounds, while incubation with β-naphthylisothiocyanate or phenylisothiocyanate inhibited both the enzymic and non-enzymic binding. Pretreatment of rats with phenobarbital or 3-methylcholanthrene enhanced ANIT toxicity *in vivo* and markedly increased the irreversible binding, *in vitro*. Piperonyl butoxide, SKF 525-A, disulfiram and cobaltous chloride reduced ANIT toxicity and its binding to microsomes *in vitro*. However, cycloheximide which abolished ANIT toxicity *in vivo* failed to affect the binding. Furthermore, 16-α-pregnenolone carbonitrile reduced ANIT toxicity *in vivo* but increased its enzymic binding. The results demonstrate an inconsistent correlation between toxicity of ANIT *in vivo* and its binding to microsomal proteins *in vitro* and indicate that binding *in vitro* in itself is not a valid index of ANIT-induced hyperbilirubinemia. Limited studies *in vivo* in which the irreversible binding to liver proteins was examined after oral ANIT administration indicated that the extent of binding after different pretreatments also did not parallel ANIT toxicity. These data cast doubt on the role of such binding in the pathogenesis of ANIT-induced cholestasis.

The acute administration of α-naphthylisothiocyanate (ANIT)§ to several animal species results in cessation of bile flow (cholestasis) accompanied by increases in plasma levels of cholesterol, bile acids and bilirubin [1]. These properties have stimulated interest in this compound as a useful model for the elucidation of the mechanisms of drug-induced cholestasis [1–4]. Although some of the effects of ANIT may be due to the parent compound, there is an increasing amount of evidence that its biotransformation products are responsible for its acute cholestatic effect. Pretreatment of animals with phenobarbital (PB) potentiates ANIT-induced cholestasis while SKF 525-A and cycloheximide (Cx) decrease or abolish the cholestatic response [5, 6]. A marked species variation to the hepatotoxic effects of ANIT exists [2, 7–9] probably because of differences in biotransformation.

Several investigators have suggested that toxicity elicited by certain model carcinogens and hepatotoxins is mediated through the formation of chemically reactive metabolites that bind irreversibly to tissue macro-molecules [10–12]. This mechanism has been proposed for the toxicity produced by 2-acetylaminofluorene [10], acetaminophen [13], furosemide [14], carbon disulfide [15, 16] and halobenzenes [17]. For these compounds, it has been shown that there is a relationship between the severity of the lesion and the amount of binding to hepatic macromolecules.

In vitro, ANIT has been shown to bind to bovine serum albumin [18]. Although it has been suggested that the binding is irreversible, neither the extent of binding nor its characteristics have been determined. Since the irreversible binding of ANIT to hepatic macromolecules could be related to its toxic effect on hepatocyte function, it is of interest to examine this phenomenon. Our experiments *in vivo* indicated that radioactivity from radiolabeled ANIT was bound irreversibly to rat liver proteins and that this binding predominates in the microsomal and cytosol fractions. Therefore, experiments *in vitro* were performed to determine the characteristics and the nature of the enzyme pathways responsible for this binding. We

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† Fellow of the Medical Research Council of Canada.

‡ Member of the Medical Research Council Group in Drug Toxicology.

§ Abbreviations used in the text are: ANIT, α-naphthylisothiocyanate; BNIT, β-naphthylisothiocyanate; PIT, phenylisothiocyanate; PB, phenobarbital; 3-MC, 3-methylcholanthrene; PCN, 16-α-pregnenolone carbonitrile; SKF 525-A, β-diethylaminoethyl diphenyl-propylacetate; $CoCl_2$, cobaltous chloride; and Cx, cycloheximide.

examined whether binding of ANIT and/or its metabolites to liver macromolecules could be related to the pathogenesis of cholestasis after ANIT. We also looked at the possibility that pretreatments that alter the toxicity of ANIT *in vivo* could influence the irreversible binding in a similar manner.

MATERIALS AND METHODS

Materials

Radioactive ANIT labeled with ^{14}C in the isothiocyanate moiety ([^{14}C]ANIT; sp. act. 1.05 mCi/m-mole; purity higher than 96 per cent) was supplied by International Chemical and Nuclear Corp., Irvine, CA. ^{3}H-ANIT labeled in the 4-position of the naphthalene ring (sp. act. 259.3 mCi/m-mole; more than 97 per cent pure) was purchased from New England Nuclear Corp. (Boston, MA). Nonradioactive ANIT was supplied by Eastman Kodak Co., Rochester, NY; β-naphthylisothiocyanate (BNIT) and phenylisothiocyanate (PIT) from Aldrich Chemical Co. (Milwaukee, WI). 16-α-pregnenolone carbonitrile (PCN) and cycloheximide (Cx) were generously provided by Upjohn Company of Canada (Montréal, Québec) and SKF 525-A was kindly supplied by Smith Kline & French Canada Ltd. (Montréal, Québec). All other reagents were the best available commercial grades.

Treatment of animals

Male Sprague–Dawley rats weighing 180–220 g were supplied by Bio-Breeding Farm Laboratory (Montréal, Québec) and were allowed free access to food (Purina Laboratory Chow) and water. Separate groups of animals were treated with phenobarbital (PB, 70 mg/kg, i.p. daily for 3 days) or PCN (70 mg/kg, p.o. twice daily for 3 days) and were used 24 hr after treatment. 3-Methylcholanthrene (3-MC, 20 mg/kg, i.p.) was injected 72, 60 and 48 hr before use. SKF-525-A (40 mg/kg, i.p.), piperonyl butoxide (1.3 ml/kg, i.p.), disulfiram (200 mg/kg, i.p.), cysteine (150 mg/kg, i.p.), cysteamine (150 mg/kg, i.p.) or cystamine (600 mg/kg, p.o.) was given 1 hr prior to use. Cx (2 mg/kg, i.p.) was injected either 1 or 8 hr before use. Pretreatment with cobaltous chloride ($CoCl_2$) consisted of two injections (40 mg/kg, s.c.) 48 and 24 hr prior to microsomal isolation or 24 and 0.5 hr before administration of ANIT. To study its effects on ANIT toxicity, BNIT or PIT (300 mg/kg, p.o.) was given 1 hr before ANIT. Control rats received equal volumes of vehicles (saline for PB, SKF 525-A, $CoCl_2$, Cx, cysteine, cysteamine or cystamine; carboxymethyl cellulose for PCN; and corn oil for 3-MC, disulfiram, BNIT or PIT).

Irreversible binding in vitro

Preparation of liver microsomes. The animals were decapitated; livers were removed and homogenized with a motor-driven glass–Teflon homogenizer in 2 vol. of 1.15% KCl containing 20 mM Tris–KCl buffer, pH 7.4. Homogenates were centrifuged for 20 min at 10,000 *g* in a Sorval centrifuge; the 10,000 *g* supernatant was then centrifuged for 1 hr at 105,000 *g* in a Spinco model L preparative ultracentrifuge. The supernatant cytosol fraction) was removed and the microsomal pellet was resuspended, washed in Tris–KCl buffer, and then recentrifuged for 1 hr at 105,000 *g*. The washed microsomal pellet was suspended in 0.1 M phosphate buffer, pH 7.4, and used within a few hours after preparation.

Incubation reactions. Incubations were carried out in glass vials containing 1 ml of liver microsomes (3 mg/ml); potassium phosphate buffer, pH 7.4 (300 μmoles); and EDTA (1.5 μmoles). To this mixture was added 2.0 ml of 1.15% KCl containing the following NADPH-generating system: NADP (1.0 μmole), glucose 6-phosphate (25 μmoles), glucose 6-phosphate dehydrogenase (2.0 units) and $MgCl_2$ (15 μmoles). Control vessels (minus NADPH) received 2.0 ml of 1.15% KCl in place of this system. The vessels were equilibrated for 3 min in a water bath shaker at 37°; then the reaction was initiated by the addition of the substrate: either [^{3}H]ANIT (≃ 1,600,000 dis./min/reaction vessel) or [^{14}C]ANIT (≃ 500,000 dis./min/reaction vessel) at the concentrations indicated in the tables and figures. ANIT was introduced into the reaction mixture in 20 μl methanol with vigorous shaking to assure even distribution; then incubations were carried out at 37°, under air, in a Dubnoff shaking incubator (100 cycles/min). Reactions were stopped at the desired time by the addition of 2.0 ml of 15% trichloroacetic acid (TCA).

Removal of the unbound material from microsomes. Samples were subjected to three 10-sec bursts of ultrasound; then aliquots of 1 ml (0.6 mg protein) were transferred onto a Whatman GF/A glass-fiber filter paper (2.4 cm). The precipitate was extensively washed, under light vacuum, with 5 ml of 10% TCA (three times), 80% methanol (four times), chloroform–methanol (2:1, four times), 80% methanol (two times) and ether (four times). This washing procedure generally removed all the extractable radioactivity, but if not, washing was continued until no further counts were removed. The filters were dried and transferred to a counting vial. One ml of 1.0 N NaOH was added and the protein was dissolved by shaking at 60° for 1 hr, then counted in Aquasol scintillation fluid (NEN Corp.).

The irreversibility of the binding was further established by extraction of some samples with methanol, chloroform or ether for 24 hr in a Soxhlet continuous extractor. Other samples were also dissolved in NaOH, reprecipitated with TCA and then extracted with organic solvents. Both procedures removed only small amounts (≃ 10 per cent of the bound radioactivity). It was noted, however, that if the protein precipitates in the reaction vessels were kept in contact with TCA for more than 5 or 6 days, some splitting of the bound radioactivity occurred. The samples, therefore, were processed within 2–3 days after incubation.

Irreversible binding in vivo

ANIT (^{3}H or ^{14}C, 150 mg/kg, 20–40 μCi/kg) was given orally to groups of rats pretreated with PB, PCN, SKF 525-A, Cx, saline or corn oil as described above. At 4, 12 or 24 hr after ANIT, groups of rats were killed by decapitation and samples of liver were removed, minced and homogenized with 3 vol. of 0.9% NaCl in a motor-driven glass–Teflon homogenizer. For estimation of total radioactivity in liver, a part of the homogenate was digested with Soluene (Packard Instrument Co.) and counted in Aquasol.

For quantitation of the radioactivity irreversibly bound to proteins, portions of the homogenate (1.0 ml, equivalent to 250 mg liver) were transferred to covered centrifuge tubes and proteins were precipitated by addition of 15% TCA. The tubes were centrifuged for 15 min ($\simeq 1000\,g$) and the supernatant was discarded. The protein precipitate was broken into a fine powder, resuspended in 5 ml of 10% TCA, mixed on a Vortex shaker for 3 min and centrifuged for 5 min. This process was repeated with 5 ml of 10% TCA, 80% methanol (two times), chloroform–methanol (2:1, two times) and ether (one time). The last washing was checked for radioactivity; then the extracted protein precipitate was dissolved in 1.0 M NaOH and aliquots were counted in Aquasol.

This extraction procedure was also used for determination of the binding *in vitro* early in our study. Quantitatively the results were only about 60–70 per cent of those obtained by the filtration method utilized for the removal of unbound material from microsomes.

Assessment of ANIT hepatotoxicity

This was carried out by measuring the rise in plasma bilirubin 24 hr after ANIT administration. ANIT, dissolved in corn oil, was given by gavage either at doses of 75 or 300 mg/kg; the low dose was used after inducers and the high dose after inhibitors. Blood was collected by aortic puncture in heparin-treated syringes and the plasma was separated for bilirubin determination.

Analytical and statistical methods

Radioactivity was counted in a Packard Tri-Carb scintillation counter (model 3375); quench correction was carried out by the channel ratio method. Protein concentration was determined according to Lowry *et al.* [19] using bovine serum albumin as the protein standard, and cytochrome P-450 content was estimated by the method of Omura and Sato [21]. Bilirubin concentration in plasma was measured by the method of Jendrassik and Grof [21] as modified by Nosslin [22]; the Monitor Jendrassik bilirubin kit provided by American Monitor Corp., Indianapolis, IN, was used.

The significance of the data was determined by the two-tailed Student's *t*-test. Significant differences were indicated when $P < 0.05$.

RESULTS

Effects of pretreatments on ANIT toxicity

Oral administration of ANIT to rats produces a dose-dependent cessation of bile flow which is associated with hyperbilirubinemia. Earlier studies have demonstrated that, in mice, these toxic effects can be potentiated with PB and inhibited with SKF 525-A [5]. In rats, Cx, an inhibitor of protein synthesis, abolishes the hyperbilirubinemic and cholestatic effects of ANIT [6]. In the present study, the effects of these, and other, inducers and inhibitors on ANIT toxicity were examined. Since the time course of hyperbilirubinemia almost parallels that of cholestasis [8], the ANIT response was assessed by the rise in plasma bilirubin. As shown in Table 1, PB considerably increased the hyperbilirubinemic effect of ANIT. A marked potentiation was also exhibited after 3-MC but, as reported earlier [23], PCN reduced the response to ANIT.

Table 1. Effects of pretreatment with inducers, inhibitors, sulfhydryl compounds and ANIT analogs on ANIT-induced hyperbilirubinemia*

Pretreatment	ANIT toxicity (hyperbilirubinemia) (mg bilirubin/100 ml plasma) Dose (75 mg/kg, p.o.)	Dose (300 mg/kg, p.o.)
None	1.12 ± 0.18	3.54 ± 0.19
Inducers		
PB	3.51 ± 0.17†	
3-MC	3.90 ± 0.22†	
PCN	0.46 ± 0.12‡	1.20 ± 0.24‡
Inhibitors		
SKF-525-A		0.99 ± 0.48‡
Pip. butoxide		0.76 ± 0.23‡
Disulfiram		1.11 ± 0.35‡
$CoCl_2$		0.38 ± 0.14‡
Cx (1-hr pretreatment)		0.33 ± 0.10‡
Cx (8-hr pretreatment)		0.41 ± 0.17‡
SH compounds		
Cysteine		1.28 ± 0.25‡
Cysteamine		0.87 ± 0.28‡
Cystamine		0.60 ± 0.22‡
ANIT analogs		
BNIT	0.57 ± 0.13‡	0.93 ± 0.20‡
PIT	1.34 ± 0.23	3.05 ± 0.38

* Mean ± S.E. of four to eight experiments.
† Significantly higher than non-treated rats.
‡ Significantly lower than non-treated rats.

Inhibitors of the mixed-function oxidase system (SKF 525-A, piperonyl butoxide and disulfiram) decreased the ANIT-induced hyperbilirubinemia; the ANIT response was also abolished by $CoCl_2$ or Cx pretreatment. Two sulfhydryl-containing compounds, cysteine and cysteamine, as well as the amino disulfide, cystamine, were also tested and were found to be effective in reducing the hyperbilirubinemia induced by ANIT.

The two ANIT analogs, BNIT and PIT, do not produce the hyperbilirubinemic effects in rats even after near-lethal doses (unpublished observations). The possibility arose that pretreatment of rats with these compounds could modify ANIT-induced hyperbilirubinemia. BNIT was found to reduce ANIT toxicity, while PIT has no observable effect (Table 1).

Binding of ANIT to microsomal proteins

Different concentrations of either [^{3}H]ANIT or [^{14}C]ANIT were incubated (in air) for various lengths of time with rat liver microsomes in the absence and in presence of an NADPH-generating system. Similar experiments were performed with microsomes obtained from PB-treated rats. The amount of non-extractable (bound) radioactivity was dependent on the ANIT concentration and on the time of incubation. Figure 1 depicts the microsomal protein binding as a function of time. Most of the experiments were carried out using 5-, 15- and 30-min incubation periods, but since the results were quantitatively similar, we report only the data obtained after 15-min incubations. ANIT binding was also dependent on microsomal protein concentration (Fig. 2). The binding was relatively linear up to a concentration of 1.0 mg/ml. Therefore, all reactions were carried out using a microsomal concentration of 1 mg/ml.

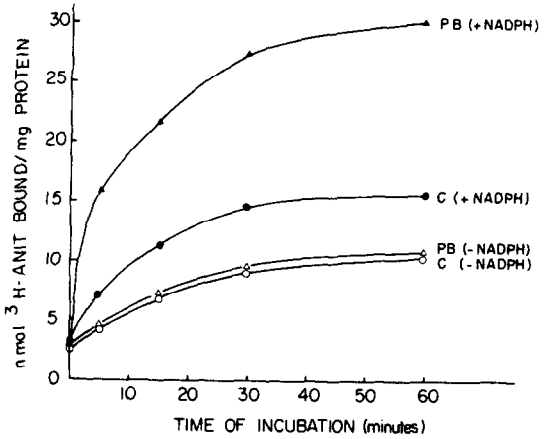

Fig. 1. Time dependence of the irreversible binding of [^{3}H]ANIT (0.1 mM) to rat liver microsomal proteins (1 mg/ml) isolated from non-treated and PB-treated rats. Incubations were carried out either in the absence or presence of the NADPH-generating system as described in Materials and Methods. Values are the means of four determinations.

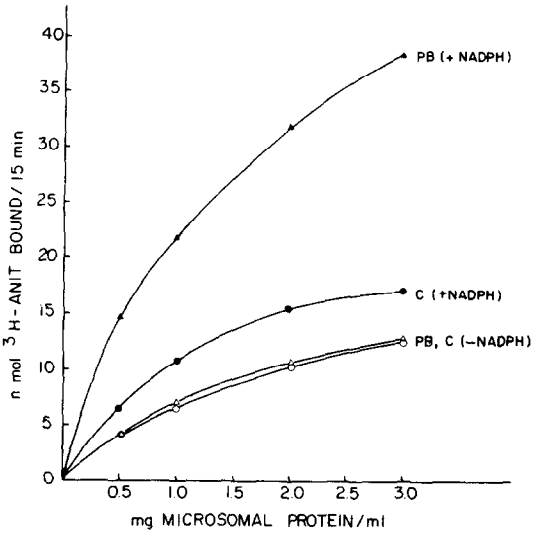

Fig. 2. Irreversible protein binding of [^{3}H]ANIT (0.1 mM) to rat liver microsomes. Incubations were performed in the absence or presence of the NADPH-generating system for a period of 15 min. Binding showed a dependence on the microsomal protein concentration. Values are the means of four experiments.

In the absence of NADPH, binding also occurred. The non-enzymic nature of this binding (−NADPH) was indicated by the increase of binding when the temperature was increased (50°). In contrast, the binding which occurred in the presence of NADPH (+NADPH) was decreased or abolished at low (5°) and at high (50°) temperatures. This enzymic binding (NADPH-dependent) was also reduced by alterations of the pH from an optimum of 7.3 to 7.9. Binding to microsomes from PB-treated rats behaved similarly but the difference between the enzymic and non-enzymic binding was much greater.

Table 2 shows some of the requirements for the enzymic binding to microsomal protein. The reaction required oxygen and NADPH, was abolished in nitrogen and was inhibited in carbon monoxide atmospheres. When non-labeled ANIT was added immediately before the incubation (0 min), both the enzymic and non-enzymic binding were reduced. However, when it was added after 10 min of incubation, it had very little effect on the binding. Incubation of the cytosol fraction (105,000 *g* supernatant) with labeled ANIT resulted in binding which was almost equal in the presence or absence of NADPH (Table 2). This suggests that the enzyme system was located only in the microsomal fraction. When the 10,000 *g* fraction, which contains cytosol and microsomes, was used as the enzyme source, the NADPH-dependent binding was much lower than that obtained with microsomes alone.

Effects of mixed-function oxidase enzyme inducers on the binding

The binding of ANIT *in vitro* by hepatic microsomes from rats pretreated with PB (Table 3) was compared to the binding by microsomes from non-treated rats (Table 2). PB considerably increased the enzymic binding without having any effect on the non-enzymic binding. In atmospheres of nitrogen or carbon monoxide the enzymic binding was strongly inhibited. Non-labeled ANIT, added before the start of incubation, reduced both the non-enzymic and enzymic binding. There were no differences in the binding to the 105,000 *g* supernatant fraction obtained from non-treated or from PB-treated rats. The NADPH-dependent binding to the 10,000 *g* fractions was increased but the increase was not as marked as that observed with the microsomal 105,000 *g* pellet.

3-MC potentiated the hepatotoxic effect of ANIT (Table 1). The data in Table 4 demonstrate that 3-MC

Table 2. Conditions for the irreversible binding *in vitro* of [^{3}H]ANIT and [^{14}C]ANIT to liver microsomes isolated from non-treated rats*

Incubation mixture	[^{3}H]ANIT (nmoles bound/mg protein/15 min)		[^{14}C]ANIT (nmoles bound/mg protein/15 min)	
	−NADPH	+NADPH	−NADPH	+NADPH
105,000 *g* Pellet (air)	6.75 ± 0.52	11.10 ± 0.71	7.21 ± 0.48	11.69 ± 0.81
Nitrogen (100%)	5.77 ± 0.41	5.49 ± 0.68†	7.03 ± 0.56	6.83 ± 0.78†
Carbon monoxide (90%)	6.73 ± 0.57	7.44 ± 0.82†	6.58 ± 0.62	7.04 ± 0.68†
Plus ANIT (0.1 mM, 0 min)	3.70 ± 0.63†	4.17 ± 0.58†	4.11 ± 0.32†	5.68 ± 0.40†
Plus ANIT (0.1 mM, 10 min)	6.36 ± 1.10	9.91 ± 1.32	6.21 ± 0.86	10.13 ± 1.04
105,000 *g* Supernatant (air)	7.12 ± 0.86	7.09 ± 0.66†	7.56 ± 0.63	7.81 ± 0.39†
10,000 *g* Supernatant (air)	6.67 ± 1.03	7.93 ± 1.26†	7.31 ± 0.82	8.45 ± 1.31†

* Mean ± S.E. of four to six experiments.
† Significantly lower than values obtained from the incubation with the 105,000 *g* pellet (air).

Table 3. Binding of [^{3}H]ANIT and [^{14}C]ANIT to liver microsomes isolated from PB-treated rats*

Incubation mixture	[^{3}H]ANIT (nmoles bound/mg protein/15 min)		[^{14}C]ANIT (nmoles bound/mg protein/15 min)	
	−NADPH	+NADPH	−NADPH	+NADPH
105,000 *g* Pellet (air)	7.10 ± 0.54	21.62 ± 2.40	6.87 ± 0.44	22.67 ± 3.01
100% Nitrogen	6.82 ± 0.39	6.93 ± 1.31†	7.14 ± 0.56	7.94 ± 0.68†
90% Carbon monoxide	6.92 ± 0.83	7.10 ± 0.99†	6.83 ± 0.73	8.21 ± 1.35†
Plus ANIT (0.1 mM, 0 min)	4.03 ± 0.45†	7.52 ± 1.24†	4.87 ± 0.61†	9.11 ± 1.56†
Plus ANIT (0.1 mM, 10 min)	6.63 ± 0.58	18.38 ± 2.06	6.34 ± 0.74	17.54 ± 1.35†
105,000 *g* Supernatant (air)	7.38 ± 0.69	7.58 ± 1.05†	8.34 ± 0.58	8.72 ± 0.98†
10,000 *g* Supernatant (air)	6.95 ± 1.03	11.83 ± 2.4†	8.16 ± 0.97	12.15 ± 2.10†

* Average of four to six experiments.
† Significantly lower than values obtained from the incubation with the 105,000 *g* pellet (air).

increased the enzymic binding of ANIT to the same extent as PB. However, PCN, which protects against the hyperbilirubinemic effect of ANIT (Table 1), also increased the enzymic binding of radioactivity to microsomes (Table 4). After treatment with PB, 3-MC and PCN, the cytochrome P-450 contents of the microsomes were increased to 170, 130 and 150 per cent, respectively, over control values; these increases did not correlate with the higher elevation of the enzymic binding of ANIT.

Inhibition of binding by mixed-function oxidase inhibitors

SKF 525-A, piperonyl butoxide, disulfiram and $CoCl_2$ known inhibitors of drug metabolism, decreased or abolished the ANIT-induced hyperbilirubinemia (Table 1). When given *in vivo* prior to sacrifice, they strongly inhibited the binding of ANIT *in vitro* (Table 5). However, when microsomes from Cx-treated rats were incubated with ANIT, the binding was not affected. When added to the incubation mixtures at concentrations of 0.5 mM, SKF 525-A, piperonyl butoxide and disulfiram abolished the enzymic binding of ANIT (Table 6). Disulfiram also significantly reduced the non-enzymic binding. When Cx (10–30 mg) was added to the incubation, the binding was not different from that obtained when no inhibitor was added (Table 6). The microsomal cytochrome P-450 content was only significantly decreased after treatment with $CoCl_2$ (63 per cent of controls); Cx caused a small, non-significant decrease when given 8 hr before sacrifice.

Kinetics of the binding

The changes in binding observed after the treatments *in vivo* with inducers and inhibitors were demonstrated at different substrate concentrations. Figure 3 shows a double reciprocal plot of the enzymic binding of [^{3}H]ANIT to rat liver microsomal proteins obtained from non-treated rats and rats treated with different inducers and inhibitors. Reciprocal binding velocities were plotted against reciprocal substrate concentrations, and the kinetic parameters of the Michaelis–Menten equation were calculated according to Lineweaver and Burk [24]. The Michaelis constant (K_m) for non-treated rats was

Table 4. Binding of [^{3}H]ANIT and [^{14}C]ANIT to microsomes obtained from 3-MC- and PCN-treated rats*

Source of microsomes	NADPH	[^{3}H]ANIT (nmoles bound/mg protein/15 min)	[^{14}C]ANIT (nmoles bound/mg protein/15 min)
Normal rats	−	6.75 ± 0.52	7.21 ± 0.48
	+	11.10 ± 0.71	11.69 ± 0.87
3-MC-treated rats	−	6.93 ± 0.71	6.77 ± 0.75
	+	22.0 ± 2.83†	23.54 ± 2.16†
PCN-treated rats	−	6.35 ± 0.86	7.16 ± 0.81
	+	14.13 ± 1.14†	13.79 ± 1.78†

* Mean ± S. E. of four to six determinations.
† Significantly higher than normal rats.

Table 5. Effect of treatment with inhibitors of drug metabolism and protein synthesis on the binding of ANIT to rat liver microsomes*

Treatment	[^{3}H]ANIT (nmoles bound/mg protein/15 min)		[^{14}C]ANIT (nmoles bound/mg protein/15 min)	
	−NADPH	+NADPH	−NADPH	+NADPH
Vehicle	6.91 ± 0.43	11.03 ± 0.60	7.18 ± 0.44	11.47 ± 0.57
SKF 525-A	6.60 ± 0.79	7.01 ± 0.94†	6.54 ± 0.81	8.01 ± 0.75†
Pip. butoxide	6.57 ± 0.55	8.11 ± 0.48†	7.37 ± 0.87	8.43 ± 0.64†
Disulfiram	6.13 ± 0.84	7.98 ± 0.69†	6.03 ± 0.56	7.64 ± 0.93†
$CoCl_2$	6.26 ± 0.47	7.06 ± 0.55†	6.50 ± 0.41	7.53 ± 0.49†
Cx (1-hr pretreatment)	7.01 ± 0.64	11.47 ± 1.13	7.51 ± 0.98	12.30 ± 1.05
Cx (8-hr pretreatment)	6.89 ± 0.51	11.58 ± 0.89	6.84 ± 0.52	11.38 ± 0.91

* Average of three to six experiments.
† Significantly lower than non-treated rats.

Table 6. Inhibition of ANIT binding by addition of various inhibitors to the incubation mixtures*

Inhibitor	NADPH	Normal rats		PB-treated rats	
		[^{3}H]ANIT (nmoles bound/mg protein/15 min)	[^{14}C]ANIT (nmoles bound/mg protein/15 min)	[^{3}H]ANIT (nmoles bound/mg protein/15 min)	[^{14}C]ANIT (nmoles bound/mg protein/15 min)
None (solvent)	–	6.36 ± 0.50	7.01 ± 0.63	6.84 ± 1.04	7.13 ± 0.86
	+	10.83 ± 1.02	11.24 ± 0.94	20.87 ± 3.14	21.11 ± 2.16
Cx (10–30 μg)	–	6.45 ± 0.31	6.87 ± 0.41	6.57 ± 0.82	7.48 ± 0.74
	+	10.86 ± 1.09	10.70 ± 1.10	21.39 ± 2.81	20.91 ± 1.93
SKF 525-A (0.5 mM)	–	6.62 ± 0.46	6.88 ± 0.55	7.36 ± 0.41	6.88 ± 0.39
	+	4.63 ± 0.96†	6.51 ± 0.82†	9.88 ± 1.63†	10.42 ± 2.13†
Piperonyl butoxide (0.5 mM)	–	5.22 ± 0.68	7.35 ± 0.34	6.35 ± 6.81	6.80 ± 0.27
	+	6.85 ± 1.17†	8.01 ± 0.76†	10.17 ± 1.98†	11.65 ± 2.06†
Disulfiram (0.05 mM)‡	–	3.66 ± 0.83†	4.53 ± 0.61†	5.03 ± 0.54†	4.72 ± 0.62†
	+	3.90 ± 0.78†	4.47 ± 0.86†	8.83 ± 1.82†	9.07 ± 1.74†

* Mean ± S. E. of three to five experiments.
† Significantly lower than values obtained in absence of inhibitors.
‡ Disulfiram was added to incubation mixtures as an ethanolic (30 mM) solution.

0.14 mM and the maximum velocity (V_{max}) was 0.69 nmole/mg of protein/min. Pretreatment with 3-MC and PB markedly increased the rate of binding reaction to 1.43 and 1.33 nmoles/mg of protein/min respectively. Apparent K_m was decreased in both cases to 0.05 mM. PCN treatment also increased the velocity of the reaction (V_{max} 0.95) and decreased K_m to 0.08 mM. Conversely, pretreatment with piperonyl butoxide decreased V_{max} (0.39) and increased K_m (0.27). Cx had no effect on both parameters (Fig. 3).

Inhibition of binding in vitro *with BNIT and PIT*

In rats, both BNIT and PIT did not elicit the hyperbilirubinemic effects of ANIT. Addition of both compounds in concentrations of 0.1 mM decreased both the enzymic and non-enzymic binding of ANIT to microsomes (Table 7). Only BNIT inhibited the hyperbilirubinemic effect of ANIT (Table 1).

Inhibition of the binding with glutathione and precursor

The effects of three sulfhydryl-containing compounds, cysteine, cysteamine and glutathione, on the binding of ANIT were also examined. At concentrations of 0.5 mM, the three compounds markedly inhibited or abolished the binding. The amino-di-sulfide, cystamine, demonstrated a similar effect (Table 8). Since this suggested that the reactive metabolite of ANIT could be an epoxide, ANIT was incubated in the presence of trichloropropene oxide, a non-competitive inhibitor of epoxide hydrase. It has been

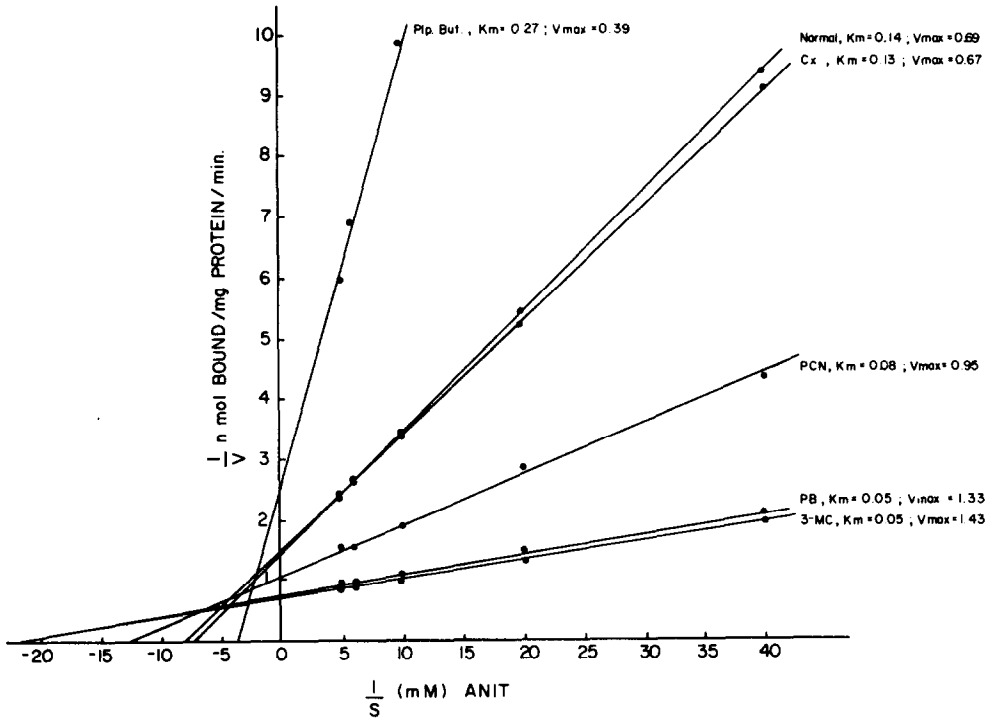

Fig. 3. Michaelis–Menten plots of the enzymic binding of [^{3}H]ANIT to rat liver microsomal proteins (1 mg/ml). Incubations were carried out with various amounts of [^{3}H]ANIT for a period of 15 min. Velocities (nmoles bound/mg protein/min) were calculated from the difference between values obtained in the presence and in the absence of the NADPH-generating system. Values are the means of four to six incubations.

Table 7. Effects of β-naphthylisothiocyanate (BNIT) and phenylisothiocyanate (PIT) on the binding of [^{3}H]ANIT and [^{14}C]ANIT*

Incubation mixture	NADPH	Normal rats		PB-treated rats	
		[^{3}H]ANIT (nmoles bound/mg protein/15 min)	[^{14}C]ANIT (nmoles bound/mg protein/15 min)	[^{3}H]ANIT (nmoles bound/mg protein/15 min)	[^{14}C]ANIT (nmoles bound/mg protein/15 min)
Plus solvent (methanol)	−	6.87 ± 0.46	6.76 ± 0.36	6.93 ± 0.53	6.54 ± 0.64
	+	11.41 ± 0.97	10.98 ± 1.21	22.04 ± 3.15	20.97 ± 2.70
Plus BNIT (0.1 mM)	−	3.99 ± 0.38†	3.72 ± 0.45†	4.31 ± 0.31†	3.76 ± 0.51†
	+	6.17 ± 0.86†	5.93 ± 0.37†	13.03 ± 2.63†	11.94 ± 1.80†
Plus PIT (0.1 mM)	−	4.30 ± 0.61†	4.21 ± 0.57†	3.75 ± 0.63†	4.11 ± 0.71†
	+	5.62 ± 0.93†	6.31 ± 0.51†	12.47 ± 1.87†	12.81 ± 2.11†

* Average of three to five determinations.
† Significantly lower than control values.

shown that when this inhibitor was incubated with imipramine, it increased its irreversible binding to rat liver microsomes [25]; with ANIT, however, the binding was decreased (Table 8).

Irreversible binding in vivo

Limited experiments were carried out after administration of [^{3}H]ANIT to rats pretreated with PB, SKF 525-A, Cx or PCN and to control rats. The animals were killed at different intervals and their livers examined for total content of ^{3}H or the amount of ^{3}H irreversibly bound to proteins. It can be seen from Table 9 that both total and bound ^{3}H continued to rise from 4 to 24 hr, but that the increase in bound radioactivity was greater as reflected in the values for per cent bound. Pretreatment of rats with PB significantly increased the bound radioactivity at 4 and 12 hr. Liver content declined more rapidly than in controls but the bound radioactivity persisted. On the other hand, pretreatment of rats with SKF 525-A resulted in decreases in both liver content and the amount of ^{3}H bound. The bound radioactivity was significantly lower than in controls at all times examined. Decreases in liver content were also found after

Table 8. Effects of sulfhydryl-containing compounds and trichloropropene oxide on the binding of ANIT*

Compound added	[^{3}H]ANIT (nmoles bound/mg protein/15 min)		[^{14}C]ANIT (nmoles bound/mg protein/15 min)	
	−NADPH	+NADPH	−NADPH	+NADPH
None (solvent)	6.36 ± 0.50	10.83 ± 1.02	7.01 ± 0.44	11.24 ± 0.98
Cysteine (0.5 mM)	5.98 ± 0.91	6.95 ± 0.83†	6.07 ± 0.52	7.41 ± 0.87†
Cysteamine (0.5 mM)	6.01 ± 0.57	8.10 ± 0.94†	6.46 ± 0.65	8.32 ± 0.68†
Cystamine (0.5 mM)	6.31 ± 0.87	7.82 ± 0.74†	6.53 ± 0.78	8.50 ± 0.74†
Glutathione (0.5 mM)	5.08 ± 0.60	7.23 ± 0.61†	5.91 ± 0.84	7.67 ± 0.91†
Trichloropropene oxide (2.0 mM)	5.01 ± 0.38	8.40 ± 0.68†	5.43 ± 0.42	9.02 ± 0.61†

* Mean ± S. E. of three to five experiments.
† Significantly lower than control values.

Table 9. Irreversible binding *in vivo* of [^{3}H]ANIT and [^{14}C]ANIT (150 mg/kg, p.o.) to liver proteins of pretreated rats*

Label	Time after ANIT (hr)		Pretreatment				
			Control	PB	SKF 525-A	Cx	PCN
^{3}H	4	Liver content	95.0 ± 6.0	105.0 ± 8.0	67.0 ± 4.0†	65.0 ± 6.0†	74.0 ± 8.0
		Amount bound	7.8 ± 0.5	17.8 ± 0.9‡	3.1 ± 0.2†	6.1 ± 0.7	9.2 ± 0.8
			(8.2)§	(16.9)	(4.6)	(9.3)	(12.4)
	12	Liver content	102.0 ± 9.0	95.0 ± 4.0	91.0 ± 5.0	72.0 ± 8.0†	121.0 ± 9.0
		Amount bound	11.2 ± 0.7	20.9 ± 1.2‡	6.6 ± 0.5†	10.0 ± 0.8	20.3 ± 1.2‡
			(10.9)	(22.0)	(7.2)	(13.8)	(16.7)
	24	Liver content	131.0 ± 11.0	76.0 ± 5.0†	104.0 ± 8.0	117.0 ± 11.0	85 ± 7.0†
		Amount bound	22.3 ± 1.1	21.3 ± 0.4	12.5 ± 1.0†	23.9 ± 1.4	22.9 ± 1.5
			(17.0)	(28.0)	(12.1)	(20.4)	(26.9)
^{14}C	4	Liver content	78.0 ± 6.0	91.0 ± 5.0	66.0 ± 5.0	49.0 ± 6.0†	68.0 ± 6.0
		Amount bound	15.6 ± 1.3	27.2 ± 2.0‡	6.5 ± 0.4†	11.8 ± 0.9	17.7 ± 1.9
			(20.0)	(29.9)	(9.8)	(24.0)	(26.1)
	12	Liver content	97.0 ± 5.0	62.0 ± 5.0†	82.0 ± 9.0	63.0 ± 8.0†	91.0 ± 6.0
		Amount bound	25.2 ± 1.9	26.7 ± 1.7	13.9 ± 1.4†	20.2 ± 1.5	27.3 ± 1.4
			(25.9)	(43.0)	(16.9)	(32.0)	(30.0)
	24	Liver content	91.0 ± 8.0	57.0 ± 6.0†	101.0 ± 8.0	81.0 ± 7.0	73.0 ± 5.0
		Amount bound	28.2 ± 2.1	26.3 ± 1.3	20.2 ± 2.1†	25.9 ± 2.7	27.8 ± 2.6
			(30.9)	(46.1)	(20.0)	(31.9)	(38.0)
Plasma bilirubin at 24 hr (mg/100 ml)			2.7 ± 0.15	4.5 ± 0.14‡	0.72 ± 0.20†	0.36 ± 0.07†	0.89 ± 0.23†
Correlation between binding and toxicity				+	+	−	−

* Mean ± S. E. of three to four. Liver content and amount bound are measured in μg/g of liver.
† Significantly lower than controls.
‡ Significantly higher than controls.
§ Values in parentheses indicate the per cent of amount irreversibly bound as related to the liver content at the time of sacrifice.

pretreatment of rats with Cx. However, the amount of ^3H bound was not significantly reduced and the percentage bound was slightly higher than in controls. PCN caused a significant increase in bound ^3H only at 12 hr. The percentage bound to proteins was not lower than in controls at all times examined.

When [^{14}C]ANIT was given in place of [^{3}H]ANIT, it was noted (Table 9) that the amount of ^{14}C bound was higher than the amount of ^3H bound. This is in contrast to the observations made *in vitro* where both labels were found to bind to the same extent. Pretreatment with inducers and inhibitors altered the binding in a manner similar to that observed after administration of [^{3}H]ANIT. In these experiments, serum bilirubin concentrations were measured at 24 hr. As expected, PB enhanced the ANIT response, whereas SKF 525-A, Cx and PCN reduced it (Table 9).

DISCUSSION

There is increasing interest in the role of irreversible binding of chemicals and drugs to tissue macromolecules as a possible mechanism for the development of different forms of toxicity. This is supported by the finding that irreversible binding of reactive metabolites to macromolecules accompanies the toxicity of several chemicals which require metabolic activation for their toxicity [10–12]. It has been postulated that ANIT metabolism to toxic product(s) is crucial for its cholestatic response [2, 5], and studies reported here (see Table 1) lend support to this view.

The data presented in this paper also indicate that ANIT (or its metabolites) binds to hepatic proteins *in vitro* and *in vivo*. Binding *in vitro* to microsomal and cytosolic proteins which occurs in the absence of NADPH probably requires no metabolic activation. In the presence of NADPH, the binding to the microsomal pellet is considerably increased. This NADPH-dependent binding involves an enzyme system which is probably located only in the microsomal fraction. It is stimulated by PB treatment (see Table 3) and inhibited by SKF 525-A (see Tables 5 and 6) and carbon monoxide (see Tables 2 and 3). These results indicate that the NADPH-dependent binding requires the conversion of ANIT to a metabolite formed oxidatively in hepatic microsomes by a cytochrome P-450 mixed-function oxidase system.

Failure of non-labeled ANIT, added after initiation of the incubation reaction, to displace the bound radioactivity further indicates that biotransformation of ANIT precedes the binding and that binding is irreversible.

When the 10,000 *g* supernatant (microsomes plus cytosol) was used as source of enzyme, binding was lower than that observed with microsomes alone. Probably the cytosol contains other enzymes which metabolize ANIT to less active products or which decompose the active metabolites formed. Alternatively, these active metabolites may react with glutathione present in the cytosol. This reaction could be catalyzed by glutathione transferase(s) available in this fraction [26].

As shown in Table 10, pretreatment of rats with PB or 3-MC, which potentiates the hyperbilirubinemic effect of ANIT, markedly increases the irreversible NADPH-dependent binding of ANIT *in vitro*. However, the enzymic binding of ANIT to microsomes obtained from PCN-treated rats also increased considerably although PCN *in vivo* reduced ANIT toxicity. Microsomes obtained from rats treated with the inhibitors SKF 525-A, piperonyl butoxide, disulfiram or $CoCl_2$, which decrease the cholestatic effect of ANIT, similarly inhibited the extent of irreversible binding. However, treatment of rats with Cx, which totally abolishes the cholestatic and hyperbilirubinemic effects of ANIT, had no effect on the microsomal binding. Thus, the correlation between toxicity *in vivo* and binding *in vitro* is inconsistent (Table 10).

A poor correlation is also seen in the experiments carried out with BNIT and PIT. These ANIT congeners lack the hyperbilirubinemic effect in rats even when given at high doses and after treatment of the animals with enzyme inducers. Pretreatment of rats with BNIT afforded some protection against the hepatotoxic effect elicited by ANIT, and addition of BNIT to the incubation medium considerably decreased ANIT binding to microsomes. But although PIT showed no protective effect against ANIT toxicity *in vivo*, its incubation with ANIT also resulted in marked reduction of the binding. Both BNIT and PIT, as well as disulfiram, also decreased the nonenzymic binding, which suggests that these compounds could be involved in a direct reaction with microsomal protein in much the same way as ANIT.

Alteration of the amount of label irreversibly bound to liver proteins *in vivo* by pretreatment with PB or with SKF 525-A suggests that a metabolic product of ANIT is involved in the binding. This, however, does not eliminate the possibility that the parent compound could, partially at least, bind without activation. The extent of binding, as well as the per cent bound relative to the total liver content, after PB and SKF 525-A treatments correlates satisfactorily with their modifying effects on ANIT hepatotoxicity. This, however, was not the case after pretreatment with either Cx or PCN (Table 9).

After Cx, the total content of radioactivity in liver was less than controls, but the amount of label bound was not significantly different. The effect of Cx on

Table 10. Correlation between ANIT toxicity and binding *in vitro*

Pretreatment before ANIT	Hyperbilirubinemia	Binding	Correlation
PB, 3-MC	Markedly potentiated	Markedly increased	+
SKF 525-A, disulfiram, piperonyl butoxide, and cobaltous chloride	Inhibited	Markedly decreased	+
SH compounds	Inhibited	Decreased	+
BNIT	Inhibited	Decreased	+
PIT	No effect	Decreased	−
Cx	Markedly inhibited	No effect	−
PCN	Inhibited	Increased	−

total hepatic content was expected since Cx reduces the oral absorption of ANIT [27]. However, when ANIT was given i.p. after Cx, both hepatic content of the label and the amount bound were not different from the values observed in controls; yet, the protective effect of Cx on ANIT toxicity was still evident (unpublished observations).

With PCN pretreatment, which also reduces ANIT toxicity, the amount of radioactivity bound was either higher (^{3}H) or almost equal (^{14}C) to that observed in controls (see Table 9). This, in addition to the Cx experiments, demonstrates that ANIT toxicity could be inhibited or abolished without affecting the amount of ANIT and/or its metabolites irreversibly bound to proteins. These results *in vivo*, although limited, are consistent with the data obtained *in vitro* which indicate that the binding is not clearly related to the development of the cholestatic lesion after ANIT.

The finding that glutathione and its precursors inhibit the enzymic binding of ANIT *in vitro* suggests that the active metabolite which binds to microsomal protein is an alkylating agent. These are nucleophilic compounds which are capable of reacting with electrophilic alkylating agents. It has been suggested that glutathione in the body may protect the liver and other organs from attack by toxic compounds and their metabolites [28]. If this is the case with ANIT, it would be expected that depletion of hepatic glutathione might lead to enhancement and/or potentiating of ANIT toxicity. We carried out some preliminary experiments in rats in which glutathione was depleted by administration of diethylmaleate before ANIT, but we found no effect on the time course or the degree of ANIT-induced hyperbilirubinemia. In another set of experiments we measured glutathione levels in the liver of rats at different time intervals (1–48 hr) after ANIT administration, but we found no decrease in glutathione content even after administration of large doses of the hepatotoxin.

Earlier studies on ANIT metabolism *in vivo* and *in vitro* were unsuccessful in identifying any of the ANIT biotransformation products [3, 7, 29]. In this study we did not attempt to characterize the metabolite(s) involved in the binding to proteins. Probably such reactive compounds would be difficult to isolate although identifications of any of its decomposition products might shed some light on its nature. The fact that the two labels (^{3}H and ^{14}C) are bound to the same extent *in vitro* indicate that the reactive intermediate possesses both the aromatic nucleus and the isothiocyanate group. Trapping of this product with SH compounds suggests that epoxidation of the naphthalene ring or the isothiocyanate moiety may be involved. Results *in vivo*, however, demonstrated that binding of the ^{14}C label is in excess of that of the ^{3}H label. This could suggest a detachment of the isothiocyanate moiety and/or further activation and binding by means which are not available to hepatic microsomal enzymes.

Some of the results presented in this paper indicate that, under certain conditions, the rate of irreversible binding of ANIT *in vitro* parallels its toxicity *in vivo*. If these experiments had been terminated after treatment with the inducers, PB and 3-MC, and the inhibitors, SKF 525-A, piperonyl butoxide, disulfiram and $CoCl_2$ we would have concluded that a good correlation existed. However, other experiments were carried out and their results in general (see Table 9) raise doubts regarding the validity of the binding as an index of ANIT toxicity. Furthermore, recent experiments [9] have been performed to determine whether the qualitative differences in ANIT-induced cholestasis observed in several species correlate with ANIT microsomal binding *in vitro*; these experiments indicate that there is no correlation.

Although there are some attractive facets to the irreversible binding hypothesis, there are also some risks inherent in this approach. Some doubt already exists regarding the non-specific binding measured by this method as a valid measure of the specific metabolite–macromolecule interaction which might initiate a toxic reaction [31]. In the case of acetaminophen [13], furosemide[14] and bromobenzene [17], the total irreversible binding to liver proteins correlates with the extent of their necrogenic properties. However, other studies with chemical carcinogens and hepatotoxins indicate some pitfalls. The non-carcinogenic, dibenz(a,c)anthracene, along with other carcinogenic benzanthracenes, is also extensively bound to liver-soluble proteins [31]. 3-MC reduces the toxicity of carbon tetrachloride, but the irreversible binding of carbon tetrachloride to proteins and lipids is elevated [32].

In conclusion, the data presented in this study demonstrate that a reactive metabolite of ANIT is formed by the mixed-function oxidase system, and that this metabolite binds extensively to rat liver proteins. However, the results indicate no clear correlation between toxicity and binding. This suggests that this irreversible binding to hepatic proteins is probably not a key event in the pathogenesis of cholestasis after ANIT. Further studies should be carried out to determine whether binding to other hepatic macromolecules would correlate better with ANIT hepatotoxicity. This might uncover binding that is related to the toxic process.

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