Microsomal Metabolism of the Hepatotoxin α-Naphthylisothiocyanate (ANIT) Following Phenobarbital, Chlorpromazine or Actinomycin D Treatment[1] (37023)

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Previous investigations of the mechanism for α-naphthylisothiocyanate (ANIT)-induced hyperbilirubinemia have suggested that microsomal biotransformation of ANIT must precede the onset of hyperbilirubinemia (1). Direct evidence for ANIT biotransformation as well as a similarity between the disposition of (^{14}C) ANIT and/or its metabolites and temporal aspects of ANIT-induced hyperbilirubinemia has previously been reported from this laboratory (2). Furthermore, a positive correlation has been found *in vivo* between the quantitative aspects of phenobarbital and chlorpromazine potentiation of ANIT-induced hyperbilirubinemia and their effect on the rate of formation of ANIT metabolites (3). Actinomycin D, on the other hand, inhibits ANIT-induced hyperbilirubinemia, inhibits phenobarbital and chlorpromazine potentiation of ANIT-induced hyperbilirubinemia, and alters certain aspects of ANIT metabolism *in vivo* (1, 3). The present study correlates previously reported *in vivo* alteration of ANIT disposition and ANIT-induced hyperbilirubinemia (2, 3) by phenobarbital, chlorpromazine and actinomycin D with the effect of these agents on *in vitro* microsomal metabolism of ANIT.

Methods. Livers were obtained from four separate groups of pretreated male Sprague-Dawley rats (150–200 g). The rats were pretreated with either chlorpromazine (20 mg/kg, ip) or phenobarbital (60 mg/kg, ip) daily for 3 days prior to sacrifice, or with actinomycin D (100 μg/kg, ip) or actinomycin D plus phenobarbital (60 mg/kg, ip) 24 and 12 hr prior to sacrifice. Control rats were treated with saline. After sacrifice, livers were immediately excised, blotted dry, weighed, placed in cold 1.15% KCl, minced with scissors and the KCl was decanted. Each liver was individually homogenized in 2 vol of 1.15% KCl with a Potter–Elvehjem homogenizer (Teflon pestle). Cell debris, cell nuclei, and mitochondria were removed by centrifugation at 9000*g* for 20 min. The microsomal fraction was obtained by centrifugation at 100,000*g* for 60 min. The resultant pellet was resuspended to the original volume in 1.15% KCl, then centrifuged for 45 min at 100,000*g* and resuspended in an equal volume of 1.15% KCl. Protein was estimated by the method of Lowry *et al.* (4) utilizing the modifications of Peters and Fouts (5).

The actual liver microsomal incubation mixture consisted of 0.5 ml of 100,000*g* supernatant containing 5 mg of microsomal protein; glucose-6-phosphate (12.5 μmole); NADP (1.0 μmole); $MgSO_4$ (12.5 μmole); and Hepes buffer (0.1 *M* final concn). The final volume was 6 ml buffered to pH 7.35. Each incubation sample represented a different animal from a respective treatment group. An additional treatment group consisted of boiled microsomes (20 min at 100°). The substrate consisted of 20 μg of ANIT (containing tracer quantities of (^{14}C) ANIT, 0.96 μCi/mmole, labeled in the isothiocyanate moiety) dissolved in 50 μl of 95% ethanol. The incubation mixture thus contained the concentration of ANIT (4.96×10^{-5} *M*) found in the liver in *in vivo* disposition studies previously reported (2, 3).

Liver microsomal incubation was conducted

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TABLE I. Microsomal Metabolism of (^{14}C) ANIT.[a]

Treatment	(^{14}C) CO_2 (%)	(^{14}C) remaining in incubation (%)
Control	2.7 ± 0.1	93.0 ± 2.0
Phenobarbital[b]	11.9 ± 0.3[d]	87.2 ± 2.8
Chlorpromazine[b]	5.2 ± 0.4[d]	89.8 ± 1.4
Boiled[b]	0.2 ± 0.1[d]	98.4 ± 0.3
Control	2.0 ± 0.2	96.1 ± 2.3
Phenobarbital[c]	6.0 ± 0.2[d]	92.0 ± 1.8
Actinomycin D[c]	1.5 ± 0.4	96.4 ± 3.0
Actinomycin D + phenobarbital	1.5 ± 0.3	94.2 ± 3.1

[a] Values shown are the percentage of the added (^{14}C) recovered from 4 individual samples (mean ± SE). The total recoveries of (^{14}C) ([^{14}C] CO_2 plus incubation [^{14}C]) ranged between 94.6 and 98.4% (no significant difference between treatment groups).

[b] The microsomal fraction was obtained from saline treated animals or animals pretreated with phenobarbital (60 mg/kg, ip daily for 3 days prior to sacrifice) or chlorpromazine (20 mg/kg, ip daily for 3 days prior to sacrifice). Boiled microsomes were prepared by heating at 100° for 20 min.

[c] Microsomes were prepared from animals treated with phenobarbital (60 mg/kg, ip) 24 and 12 hr prior to incubation or with actinomycin D (100 μg/kg, ip) or both simultaneously.

[d] Significantly different from respective control ($p < .05$).

in 25 ml Erlenmeyer stopper flasks using a Dubnoff shaking apparatus (100 oscillations/min) at 37° for 15 min under an atmosphere of oxygen (flow rate, 1000 ml/min). Throughout the incubation period (^{14}C) CO_2 generated from ^{14}C-ANIT was collected quantitatively by means of a NaOH trapping solution contained in a separate bubbling vessel connected distal to the Erlenmeyer flask. The amount of (^{14}C) CO_2 trapped was quantitated by the method of Yeh and Woods (6). At the end of the 15 min incubation period the reaction was terminated by addition of 2.5 ml of methanol to each of the incubation beakers.

All incubation mixtures were then exhaustively extracted with methanol to remove the remaining (^{14}C). In addition, nonboiled and boiled (20 min at 100°) microsomal controls were extracted similarly with methanol immediately after addition of (^{14}C) ANIT to determine the recovery of (^{14}C) from nonincubated mixtures. An aliquot from each incubation mixture extract and from nonboiled and boiled controls extracted at zero time, was chromatographed on silica gel thin layer plates, and developed in benzene:methanol (95:5) and butanol-propanol:ammonia (5:2:2) solvent systems (2, 3).

An analysis of variance was completed on all data and analysis for statistical significance between the various treatment means was accomplished using a least significant procedure at a significant level of $p < .05$ (7).

Results and Discussion. The *in vitro* metabolism of (^{14}C) ANIT was studied utilizing an isolated rat liver microsomal fraction. Table I shows the total (^{14}C) CO_2 recovered following incubation of microsomal fractions prepared from livers obtained from the various treatment groups. Approximately 2–3% of the added ANIT was converted to CO_2 by the control microsomal fractions. In previously reported *in vivo* disposition studies approximately 20% of the administered dose was recovered as CO_2 from the expired air by 24 hr (2, 3). Incubation of microsomal fractions isolated from phenobarbital and chlorpromazine pretreated animals showed significantly greater amounts of (^{14}C) CO_2 production when compared to controls. Only trace amounts of (^{14}C) CO_2 were recovered from control microsomal fractions which had been boiled prior to incubation. In a second experiment (Table I) actinomycin D did not significantly alter (^{14}C) CO_2 production from (^{14}C) ANIT in incubations of control microsomal fractions but did block phenobarbital induction. In both experiments total recoveries of (^{14}C) [incubation mixture and (^{14}C) CO_2] were not significantly different among the various treatment groups (range 94.6–98.4%).

The (^{14}C) remaining in the incubation mixture was extracted with methanol and analyzed chromatographically. In control experiments with boiled and nonboiled microsomal fractions which were not incubated but extracted immediately after addition of (^{14}C) ANIT, recoveries of the added (^{14}C) were greater than 98%. However, chromatography

of the extracts revealed that the majority of the (^{14}C) failed to migrate as (^{14}C) ANIT. The major compoent was a single peak at the origin with less than 10% of the radioactivity remaining as free ANIT. Chromatography of extracts from incubated samples revealed several small peaks in addition to a major peak near the origin. Only trace amounts of ANIT were found. TCA precipitation and vigorous acid hydrolysis failed to modify the (^{14}C) chromatographic pattern of either the microsomal incubation or the nonincubated extracts. Preliminary experiments were subsequently done in an attempt to identify the substances binding ANIT. Purified phospholipid did not bind ANIT. Bovine albumin irreversibly bound ANIT resulting in a single peak at the origin. The binding of ANIT to albumin was not reversible with acid hydrolysis, TCA precipitation, or boiling.

The data presented indicate that the liver microsomal fraction is capable of converting ANIT added *in vitro* to CO_2. In addition, it is possible to induce the metabolic pathway leading to CO_2 production by pretreating rats with phenobarbital and chlorpromazine. The phenobarbital induction can be blocked by the simultaneous administration of actinomycin D. These results are similar to the *in vivo* disposition studies (3) in which a significant increase in expired and urinary (^{14}C) followed phenobarbtal and chlorpromazine pretreatment and actinomycin D pretreatment significantly decreased levels of expired and urinary (^{14}C). Thus, this study supports the hypothesis that phenobarbital and chlorpromazine pretreatment may enhance microsomal conversion of ANIT to a hepatotoxic metabolite and actinomycin D may be effectively blocking the induction by phenobarbital.

Spontaneous and irreversible binding of (^{14}C) ANIT to the microsomal fraction suggests another possible explanation for the mechanism for ANIT-induced hyperbilirubinemia. The hyperbilirubinemic effect of ANIT may be a consequence of binding of ANIT or its metabolites to substances within the liver parenchyma which are critical for normal hepatic biliary secretory function. Binding of ANIT and/or its metabolites with this "critical factor" may be susceptible to exogenous influences created by drugs or chemicals. Studies are currently underway to identify the metabolites of ANIT and the cellular constituents which may bind with ANIT and/or its metabolites.

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