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EFFECT OF INHIBITORS OF PROTEIN AND RIBONUCLEIC ACID SYNTHESIS ON THE ALTERATION IN BILIARY BILIRUBIN EXCRETION AND NON-ERYTHROPOIETICALLY DERIVED BILIRUBIN SYNTHESIS IN RATS AFTER α-NAPHTHYLISOTHIOCYANATE ADMINISTRATION*

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Abstract—In an attempt to explain the inhibition of α-naphthylisothiocyanate (ANIT)-induced hyperbilirubinemia by inhibitors of protein and RNA synthesis, the effect of cycloheximide, ethionine and actinomycin D on the increased bilirubin formation produced by ANIT was studied. Cycloheximide and ethionine pretreatment inhibited the ANIT-induced increase in biliary bilirubin excretion and incorporation of δ-aminolevulinic acid-^{14}C (ALA-^{14}C) into bilirubin. Cycloheximide was found to be more effective than ethionine in diminishing the stimulation of bilirubin production by ANIT. Pretreatment with actinomycin D prevented the ANIT-induced increase in biliary bilirubin excretion and produced a decreased, but irregular incorporation of ALA-^{14}C into bilirubin. β-Naphthylisothiocyanate, which does not produce hyperbilirubinemia, failed to enhance biliary bilirubin excretion. The results suggest that the enhancement of bilirubin formation by ANIT plays a role in the overall hyperbilirubinemic response seen after ANIT administration. The inhibition of the enhanced bilirubin formation by cycloheximide and ethionine may partially account for their inhibition of ANIT hyperbilirubinemia.

THE ACUTE administration of α-naphthylisothiocyanate (ANIT) in mice and rats produces hyperbilirubinemia and cholestasis.[1–3] Roberts and Plaa[4] have shown that ANIT increases biliary bilirubin content and increases the incorporation of δ-aminolevulinic acid-^{14}C (ALA-^{14}C) into bilirubin before the onset of cholestasis. The temporal aspects of these increases were compatible with the onset of acute ANIT-induced hyperbilirubinemia and were suggested as a contributing factor in the subsequent elevation of plasma bilirubin concentrations.

A previous investigation in this laboratory has shown that inhibitors of protein and RNA synthesis diminish ANIT-induced hyperbilirubinemia and cholestasis.[5] The agents differed in their capacity to protect against this hepatotoxic response to ANIT. Cycloheximide afforded the greatest degree of protection, ethionine was intermediate, and actinomycin D only partially diminished the response. The mechanism by which this inhibition occurs remains unresolved. However, it has also

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been shown[6] that the administration of these inhibitors does not abolish ANIT-induced sulfobromophthalein retention and prolongation of pentobarbital hypnosis, two dose-dependent manifestations of ANIT toxicity which appear many hours before the onset of hyperbilirubinemia and cholestasis.[2] Therefore, the purpose of the present study was to examine the effect of these inhibitors on ANIT-induced stimulation of bilirubin excretion and synthesis, a response which also occurs many hours before the onset of cholestasis. To elucidate further the role of increased bilirubin production in the hyperbilirubinemic response, the effect of β-naphthylisothiocyanate (BNIT) on bilirubin excretion and synthesis was investigated as well.

METHODS

Male Sprague–Dawley rats (300–400 g), maintained on Purina Chow and tap water were used throughout the study. ANIT (150 mg/kg) or BNIT (300 mg/kg) was suspended in 1·5% carboxymethylcellulose (CMC) and administered by gavage. Control animals received an identical treatment with 1·5% CMC. Actinomycin D (100 μg/kg) and cycloheximide (2 mg/kg) were administered intraperitoneally 1 hr prior to the administration of ANIT. Ethionine (400 mg/kg) was administered intraperitoneally 2 hr prior to ANIT. Control animals received identical treatment with saline. δ-Aminolevulinic acid-4-^{14}C-HCl was given intravenously (0·72 μCi/kg) dissolved in 0·9% NaCl.

The study of the incorporation of ALA-^{14}C was carried out as previously described.[4] At appropriate time intervals after the respective treatments, rats were anesthetized with pentobarbital (45 mg/kg i.p.). The bile duct and femoral vein were exposed, and the bile duct was cannulated with PE-10 tubing. Saline (1 ml/hr) was administered intraperitoneally during the experiment to compensate for the loss of bile. A 30-min equilibration period preceded the intravenous administration of ALA-^{14}C. ALA-^{14}C was always administered 2 hr after ANIT or BNIT. Bile samples were collected hourly for a period of 3 hr under ice and analyzed immediately for volume and bilirubin content. Bile volume was estimated directly with a graduated pipette. Biliary bilirubin content was measured by the procedure of Jendrassik and Grof[7] as modified by Nosslin.[8,*] Bilirubin-^{14}C was estimated employing the extraction and a modification of the chromatographic purification procedures previously described.[4] An aliquot of diazotized bile containing azopigments was subjected to descending paper chromatography using methyl ethyl ketone–propionic acid–water–isopropanol (50:26:30:1) as the solvent system. The chromatograms were run for 15 hr at 0°. The solvent system yielded an R_f of 0·46 for ALA, 0·58 for conjugated diazotized bilirubin and 0·69 for unconjugated diazotized bilirubin. All chromatography was completed within 24 hr of sample collection. Portions of the chromatograms containing the azopigment were cut out and placed in counting vials containing 5 ml of acid–alcohol (97 ml of 95% ethanol and 3 ml of concentrated HCl). After approximately 24 hr, 15 ml of a phosphor solution was added to each vial. The phosphor solution consisted of 270 ml phenethylamine, 270 ml absolute methanol, 1 liter toluene, 5·0 g 2,5-diphenyloxazole (PPO) and 100 mg *p-bis*[2(5-phenyloxazolyl)] benzene (POPOP). The samples were counted in a Packard Tri-Carb liquid scintillation spectrometer. Automatic external standardization was used for determining and correcting quench.

* The Monitor Jendrassik Bilirubin Kit was used; American Monitor Corp., Indianapolis, Ind.

A group Student's *t*-test was utilized for all statistical comparisons if an F-test indicated homogeneity of variances.[9] In the case of nonhomogeneity, the Mann–Whitney U test was employed.[10] A significance level of $P < 0.05$ (one-tailed) was used as a basis for rejection of the null hypothesis.

Actinomycin D was obtained from Merck–Frosst Laboratories, Kirkland, Quebec; cycloheximide from Upjohn Co. of Canada, Montreal, Quebec; ethionine from Sigma Chemical Co., St. Louis, Mo; ANIT and BNIT from Eastman Kodak, Rochester, N.Y.; δ-aminolevulinic acid-4-^{14}C-HCl, 20·9 mCi/m-mole, from New England Nuclear, Boston, Mass.; all other chemicals were of the highest purity commercially available.

RESULTS

Figure 1 depicts the effect of cycloheximide pretreatment on ALA-^{14}C incorporation in control and ANIT-treated animals. At the end of each hr, beginning 2 hr after ANIT administration, a sample of bile was taken and analyzed for bilirubin-^{14}C radioactivity and bilirubin content. As previously reported,[4] ANIT increased the incorporation of ALA-^{14}C into bilirubin. Cycloheximide, administered 1 hr before ANIT, blocked significantly this effect of ANIT at all three collection periods.

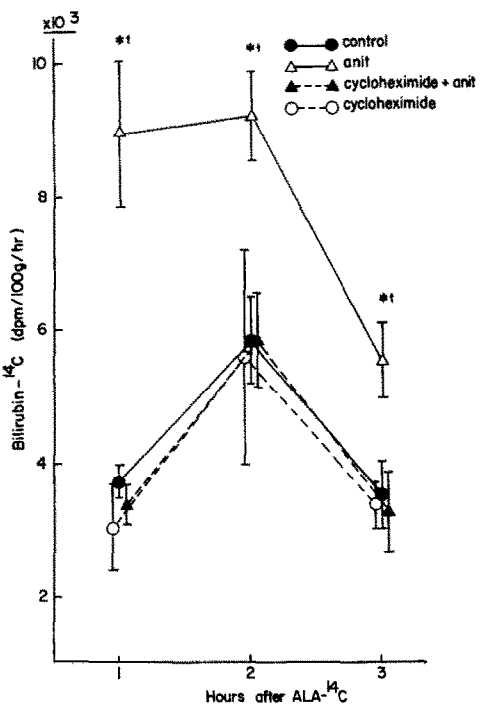

FIG. 1. Effect of cycloheximide pretreatment on ANIT-induced increase of ALA-^{14}C incorporation into bilirubin. Cycloheximide (2 mg/kg i.p.) was given 1 hr prior to ANIT (150 mg/kg p.o.). ALA-^{14}C (0·72 μCi/kg) was administered intravenously 2 hr after ANIT and bile was collected continuously for a period of 3 hr thereafter. Each point represents the mean ± S.E. for a group of four to five animals. The asterisk (*) indicates a statistically significant difference when compared with the values of control animals ($P < 0.05$). The symbol, †, indicates a statistically significant difference when compared with the values of animals receiving cycloheximide plus ANIT ($P < 0.05$).

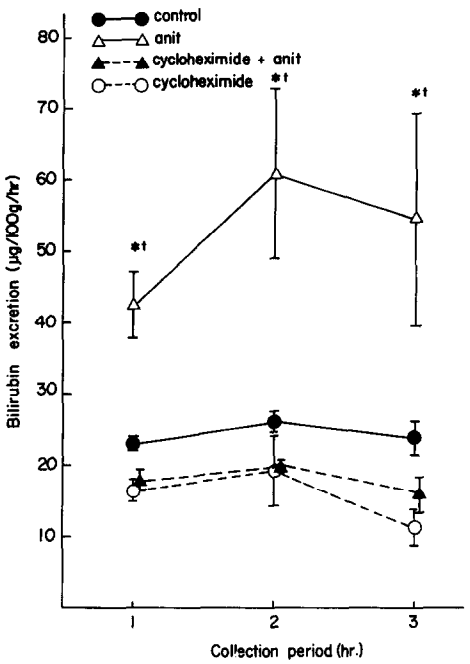

FIG. 2. Effect of cycloheximide pretreatment on ANIT-induced increase in biliary bilirubin excretion. Experimental details and symbols (*, †) are as in Fig. 1. These results were obtained from the same bile samples used to estimate ALA-^{14}C incorporation into bilirubin. Each point represents the mean ± S.E. for a group of four to five animals.

Figure 2 shows the effect of cycloheximide on biliary bilirubin excretion in control and ANIT-treated rats over a 3-hr collection period. Cycloheximide blocked the increased bilirubin excretion produced by ANIT, throughout the collection period.

Table 1 summarizes the overall effect of cycloheximide on ANIT-induced increases of ALA-^{14}C incorporation and bilirubin excretion (Figs. 1 and 2). ANIT, when

TABLE 1. SUMMARY OF THE EFFECTS OF CYCLOHEXIMIDE ON ANIT-INDUCED INCREASE IN BILIRUBIN EXCRETION AND ALA-^{14}C INCORPORATION INTO BILIRUBIN*

Groups		Total biliary bilirubin excretion (μg/100 g)	Total bilirubin-^{14}C (dis./min/100 g × 10^{-3})	% Incorporation of ALA-^{14}C
Control	(5)	73·6 ± 4·0	13·2 ± 1·3	8·2 ± 0·8
ANIT	(4)	157·9 ± 32·1†	23·7 ± 1·5†	14·8 ± 0·9†
Cycloheximide + ANIT	(4)	54·4 ± 4·3‡	13·5 ± 1·2‡	8·4 ± 0·9‡
Cycloheximide	(4)	48·8 ± 10·3	12·4 ± 1·1	7·2 ± 0·7

* The results are a summary of the 3-hr experiments depicted in Figs. 1 and 2. See Fig. 1 for experimental details. The values shown are the mean ± S.E. determined from the number of animals indicated in the parentheses.

† Significantly different from control group ($P < 0.05$).

‡ Significantly different from group receiving ANIT alone ($P < 0.05$).

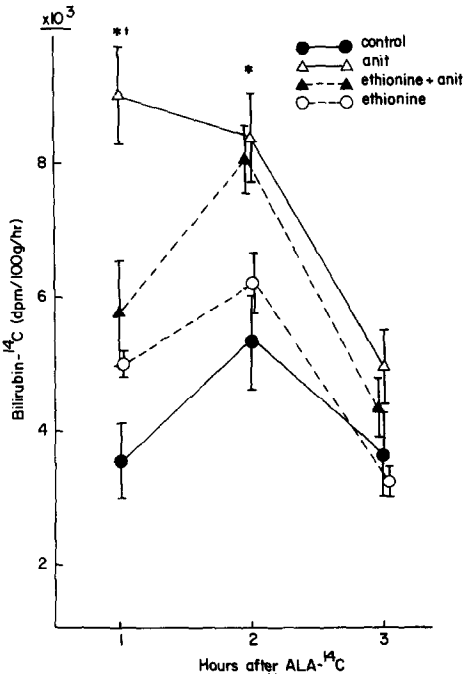

FIG. 3. Effect of ethionine pretreatment on ANIT-induced increase of ALA-^{14}C incorporation into bilirubin. Ethionine (400 mg/kg i.p.) was given 2 hr prior to ANIT (150 mg/kg p.o.). ALA-^{14}C (0·72 μCi/kg) was administered intravenously 2 hr after ANIT and bile was collected continuously for a period of 3 hr thereafter. The points for ethionine alone represent the mean ± S.E. for a group of two animals. All other points represent the mean ± S.E. for a group of five to six animals. The asterisk (*) indicates a statistically significant difference when compared with the values of the control animals ($P < 0·05$). The symbol, †, indicates a statistically significant difference when compared with the values of animals receiving ethionine plus ANIT ($P < 0·05$).

given alone, increased the percentage of incorporation of ALA-^{14}C and the amount of bilirubin-^{14}C; cycloheximide significantly reduced the increased bilirubin-^{14}C synthesis from ALA-^{14}C produced by ANIT. The biliary bilirubin excretion rate produced by cycloheximide alone was not significantly lower than that found for control animals, and the per cent incorporation of ALA-^{14}C in this group was not significantly lower than that observed in the control group.

Figures 3 and 4 depict the effect of ethionine on ANIT-induced increases of ALA-^{14}C incorporation and biliary bilirubin excretion respectively. Ethionine pretreatment was effective in reducing the incorporation of ALA-^{14}C at the first hr only; the bilirubin-^{14}C produced at the second and third hr in animals treated with ethionine and ANIT was similar to that produced by ANIT alone (Fig. 3). Ethionine pretreatment also significantly diminished the ANIT-induced increases in bilirubin excretion at all three periods of bile collection (Fig. 4).

Table 2 summarizes the overall effect of ethionine (Figs. 3 and 4) and actinomycin D on ANIT-induced increases of bilirubin synthesis and excretion. The bilirubin-^{14}C synthesis from ALA-^{14}C and biliary bilirubin excretion in the group receiving ethionine plus ANIT were significantly lower than those found for the group receiving ANIT alone. It should be pointed out that ethionine (Table 2) was not as

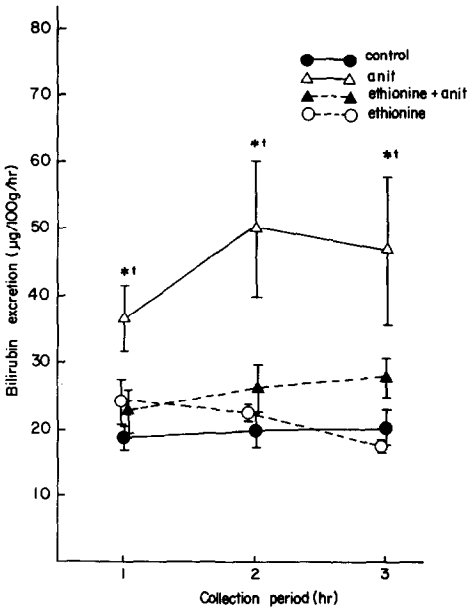

FIG. 4. Effect of ethionine pretreatment on ANIT-induced increase in biliary bilirubin excretion. Experimental details and symbols (*, †) are as in Fig. 3. These results were obtained from the same bile samples used to estimate ALA-^{14}C incorporation into bilirubin. Each point represents the mean ± S.E. for a group of two to six animals.

effective as cycloheximide (Table 1) for reducing the ANIT-induced increases in both parameters.

Actinomycin D was the least effective of the three inhibitors studied. Actinomycin D pretreatment before ANIT administration reduced the percentage of incorporation of ALA-^{14}C, but the value was not significantly different from the incorporation

TABLE 2. SUMMARY OF THE EFFECTS OF ETHIONINE AND ACTINOMYCIN D ON ANIT-INDUCED INCREASE IN BILIRUBIN EXCRETION AND ALA-^{14}C INCORPORATION INTO BILIRUBIN*

Groups		Total biliary bilirubin excretion (μg/100 g)	Total bilirubin-^{14}C (dis./min/100 g × 10^{-3})	% Incorporation of ALA-^{14}C
Control	(5)	58·9 ± 6·3	12·7 ± 1·8	7·8 ± 1·05
ANIT	(6)	133·5 ± 25·5†	21·8 ± 1·5†	13·6 ± 0·9†
Ethionine + ANIT	(5)	74·7 ± 8·5‡	18·1 ± 1·2‡	11·3 ± 0·8‡
Ethionine	(2)	64·4 ± 4·6	14·5 ± 0·8	9·05 ± 0·5
Actinomycin D + ANIT	(4)	67·4 ± 8·7‡	17·2 ± 2·6	10·7 ± 1·6
Actinomycin D	(4)	53·6 ± 4·8†	12·8 ± 0·5	8·0 ± 0·3

* The results for ethionine are a summary of the 3-hr experiments depicted in Figs. 3 and 4. See Fig. 3 for experimental details of ethionine pretreatment. Actinomycin-D (100 μg/kg i.v.) was administered 1 hr prior to ANIT (150 mg/kg p.o.). ALA-^{14}C was given 2 hr after ANIT and bile was collected continuously for a period of 3 hr. The values shown are the mean ± S.E. determined from the number of animals indicated in the parentheses.

† Significantly different from control group ($P < 0.05$).

‡ Significantly different from group receiving ANIT alone ($P < 0.05$).

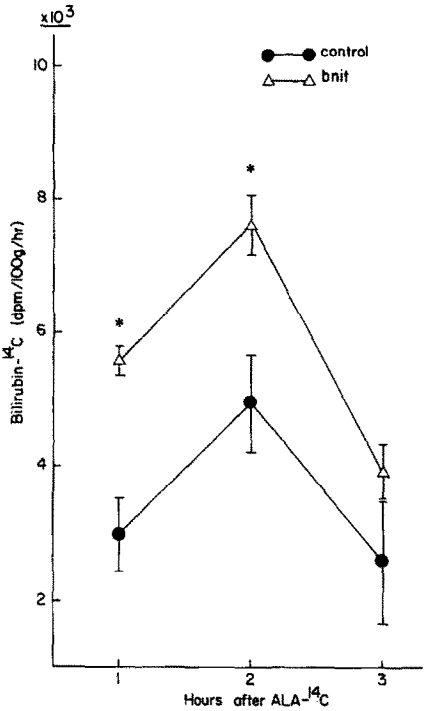

FIG. 5. Effect of BNIT on ALA-^{14}C incorporation into bilirubin. BNIT (300 mg/kg p.o.) was given 2 hr prior to ALA-^{14}C administration (0·72 μCi/kg i.v.). Bile was collected continuously after ALA administration for a period of 3 hr. The mean values $\pm$ S.E. shown were derived from three control and four BNIT-treated animals. The asterisk (*) indicates that the BNIT values are significantly different from those of controls ($P < 0.05$).

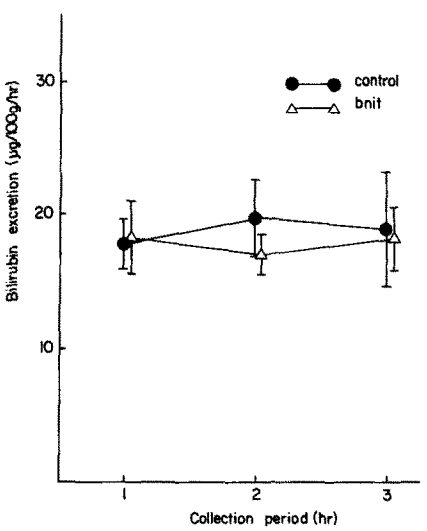

FIG. 6. Effect of BNIT on biliary bilirubin excretion. These results were obtained from the same bile samples for which ALA-^{14}C incorporation values are shown in Fig. 5. The mean values $\pm$ S.E. shown were derived from three control and four BNIT-treated animals.

found for ANIT alone. Actinomycin D did significantly diminish the increase in biliary bilirubin excretion produced by ANIT (Table 2).

Figures 5 and 6 show, respectively, the pattern of ALA-^{14}C incorporation and biliary bilirubin excretion produced by BNIT. This isomer of ANIT does not produce hyperbilirubinemia.[11] BNIT (300 mg/kg p.o.) produced an increase in the incorporation of ALA-^{14}C (Fig. 5). The 3-hr total bilirubin-^{14}C for the control animals was $10{\cdot}5 \pm 1{\cdot}8 \times 10^3$ dis./min/100 g (6·6 per cent incorporation), whereas that for the BNIT-treated group was significantly higher, $17{\cdot}1 \pm 1{\cdot}0 \times 10^3$ dis./min/100 g (10·7 per cent incorporation). As shown in Fig. 6, the biliary bilirubin excretion rate found for BNIT-treated animals was identical to that found for controls. Control animals exhibited a 3-hr mean bilirubin excretion rate of $18{\cdot}8 \pm 0{\cdot}6$ μg/100 g/hr, while that of BNIT-treated animals was $17{\cdot}9 \pm 0{\cdot}4$ μg/100 g/hr.

DISCUSSION

Prior to the onset of hyperbilirubinemia and cholestasis, ANIT has been shown to increase biliary bilirubin excretion and the formation of nonerythropoietically derived bilirubin.[4] Roberts and Plaa[12] have reported that certain drugs known to potentiate ANIT-induced hyperbilirubinemia can also increase the rate of bilirubin formation. These observations would suggest that ANIT-induced hyperbilirubinemia may in part be a consequence of an increase in bilirubin production.

Indacochea-Redmond *et al.*[5] reported that cycloheximide, ethionine and actinomycin D at doses which inhibit protein or RNA synthesis, or both, are not equally effective in depressing the hyperbilirubinemic response to ANIT. Cycloheximide pretreatment reduced ANIT-enhanced plasma bilirubin concentrations to control values; ethionine produced a marked inhibition, and actinomycin D only partially diminished the response to ANIT. In the present study, the ability of cycloheximide and ethionine to reduce ANIT-induced increases in ALA-^{14}C incorporation into bilirubin and biliary bilirubin excretion correlates well with the ability of these agents to protect against the hyperbilirubinemia produced by ANIT. This correlation provides additional evidence for a role of ANIT-induced stimulation of bilirubin production in the overall hyperbilirubinemic response.

Among the various effects of ANIT on hepatocyte function, one finds that within 2–6 hr after its administration sulfobromophthalein retention, prolongation of pentobarbital hypnosis and enhanced formation of nonerythropoietically derived bilirubin occur.[2,4,6] Indacochea-Redmond *et al.*[6] observed that cycloheximide, ethionine and actinomycin D do not affect ANIT-induced sulfobromophthalein retention or prolongation of pentobarbital hypnosis. However, the present study shows that cycloheximide and ethionine do modify the amount of nonerythropoietically derived bilirubin formed after the administration of ANIT. These data suggest that the effect of ANIT on bilirubin production is not related to the depression of hepatic microsomal enzymes known to occur[13] after ANIT administration, and which accounts for the prolongation of pentobarbital hypnosis and a prolongation in the duration of paralysis after administration of zoxazolamine.[14]

In the normal rat, most of the bilirubin appearing in the bile is derived from hemoglobin heme in senescent red blood cells. However, about 15 per cent is derived from nonerythroid sources, and the liver seems to be the principal source of this nonhemoglobin heme.[15] While studies using glycine-^{14}C incorporation into bilirubin

measure both the erythroid and nonerythroid components, ALA-^{14}C incorporation virtually measures only the nonerythroid component.[15]

The increase in biliary bilirubin observed after ANIT treatment could be due to hemolysis or to the enhanced formation of nonerythropoietic bilirubin, or to both. The observation that ANIT treatment alone resulted in enhanced incorporation of ALA-^{14}C indicates that the production of nonerythropoietic bilirubin had increased. However, while the total amount of bilirubin excreted in the rats treated with only ANIT doubled in quantity when compared to controls (Tables 1 and 2), the per cent incorporation of ALA-^{14}C also only doubled. Assuming that the size and specific activity of the precursor (ALA) pool do not change in ANIT-treated rats, one would expect that the amount of ALA-^{14}C incorporated should increase more than twice if only the nonerythroid component were involved. The results obtained could be interpreted to indicate that both erythroid and nonerythroid sources are involved in the increased biliary bilirubin excretion seen after ANIT treatment.

In their original studies, Roberts and Plaa[4] were unable to detect changes in plasma hemoglobin concentrations or in hematocrit in rats treated with ANIT. Therefore, they concluded that hemolysis had been ruled out as a major source of the ANIT-induced increase in biliary bilirubin excretion. However, the amount of hemolysis required to cause only a doubling of biliary bilirubin content would not have been detectable by the methods employed. Consequently the erythropoietic component, which also seemed to have increased after ANIT treatment, could be due to an undetectable increase in erythrocyte breakdown.

On the other hand, the increase in biliary bilirubin observed after ANIT might all be derived from nonerythroid sources, but an enlarged ALA pool masks this effect by diminishing the specific activity of the labeled ALA at the site of formation. Normally, heme is synthesized from ALA following the condensation of glycine and succinyl-CoA; this rate-limiting step, mediated by ALA synthetase, is repressed by heme.[15] Nothing is known about the effects of ANIT on this pathway. If ANIT were to enhance ALA synthetase activity or were to abolish the repressor effect of heme, enhanced endogenous ALA formation would occur, which could lead to an increased formation of bilirubin. In the type of experiments utilized in the present study, such an enhanced formation of endogenous ALA would enlarge the ALA pool and would decrease the specific activity of the effective ALA pool utilized for bilirubin formation. The nonlabeled nonerythroid bilirubin so derived would be indistinguishable from erythropoietically derived bilirubin. Therefore, it cannot be said with certainty that the increased biliary bilirubin excretion observed in ANIT-treated rats comes from both erythroid and nonerythroid sources.

Another factor which merits consideration is the hepatic storage of bilirubin. Bilirubin, prior to its excretion into the bile, resides in a hepatic storage space which is in equilibrium with the plasma compartment.[16] It is possible that ANIT treatment causes changes in bilirubin distribution, which result in a decrease in the relative storage capacity of the liver, and that this effect favors the biliary excretion of bilirubin. Bilirubin concentrations in the liver of mice after the exogenous administration of bilirubin have been shown to be reduced 24 hr after ANIT.[17] Since most of the bilirubin residing in the liver should be derived from hemoglobin breakdown, this could also account in part for an apparently increased erythroid component observed following ANIT treatment.

Obviously, the effect of ANIT on bilirubin production is not at all clear, due to the complexity of the experimental situation. Ideally, under the same experimental conditions, one would need to have information regarding the bilirubin pool, the ALA pool, the heme pool and measurements of both erythroid and nonerythroid bilirubin components. Unfortunately, in the present experiments, this information is lacking. However, the fact that the mechanisms involved in the ANIT effect are still unclear should not detract from the observation that inhibitors of protein synthesis can markedly diminish this biologic effect of ANIT.

BNIT differs from ANIT in that, in mice, BNIT does not produce hyperbilirubinemia, while ANIT does.[11] However, both substances inhibit certain hepatic microsomal enzyme systems.[13] In the present study, BNIT, like ANIT, was shown to increase ALA-^{14}C incorporation into bilirubin, but BNIT, unlike ANIT, did not increase biliary bilirubin excretion. Further differences are observed when one compares the ANIT and BNIT responses. The peak incorporation of ALA-^{14}C into bilirubin occurred within the first hr in the animals treated only with ANIT (Figs. 1 and 3), whereas the peak incorporation occurred during the second hr in the BNIT-treated and the control rats (Figs. 1, 3 and 5). The specific activities for bilirubin-^{14}C calculated from the data presented in Figs. 1–6 are given in Table 3. They indicate that the peak values for ANIT at 1 hr in the two experiments never exceeded the peak values obtained for the controls at 2 hr in the same experiments. However, with BNIT, the peak value exceeded the one obtained in control animals. The three 1-hr values for BNIT-treated rats were always larger than those obtained in controls and mirrored the pattern of ALA-^{14}C incorporation observed in control rats, whereas in animals treated only with ANIT, the specific activities calculated for the second and third hr were lower than those obtained in control animals. The means for the 1-hr ANIT values were lower than those of the respective controls, while the mean for BNIT-treated rats was larger than the control value. These data clearly indicate that the biliary bilirubin responses following BNIT administration are both qualitatively and quantitatively different from those observed with ANIT.

Since BNIT produces an increased incorporation of ALA-^{14}C into bilirubin without an increase in total biliary bilirubin excretion, it is difficult to conclude that the

TABLE 3. SPECIFIC ACTIVITIES FOR THE BILIRUBIN-^{14}C DATA PRESENTED IN FIGS. 1–6*

Experiment	Treatment		Collection period			
			1 hr	2 hr	3 hr	Mean
I	Control	(5)	161 ± 9	223 ± 22	156 ± 30	180 ± 21
	ANIT	(4)	223 ± 41	173 ± 37	117 ± 20	171 ± 31
II	Control	(5)	207 ± 48	316 ± 101	201 ± 55	241 ± 37
	ANIT	(6)	251 ± 32	192 ± 27	119 ± 13	187 ± 38
III	Control	(3)	178 ± 53	258 ± 50	142 ± 35	193 ± 35
	BNIT	(4)	328 ± 59	452 ± 19	217 ± 10	332 ± 67

* The values are expressed as dis./min/ng bilirubin, mean ± S.E., determined from the number of animals in the parentheses.

formation of nonerythropoietically derived bilirubin has actually been increased following BNIT treatment. If it had been increased, it would have to have been accompanied by a decrease in nonlabeled bilirubin in order to maintain total bilirubin excretion at control values. BNIT might enhance the flux of bilirubin from the liver to the plasma; however, BNIT does not cause hyperbilirubinemia.[11] On the other hand, the increase in bilirubin-^{14}C after BNIT treatment might be due to a diminution in the size of the effective ALA precursor pool; this would increase the specific activity of the labeled ALA incorporated into bilirubin. Obviously, here too, information regarding ALA and bilirubin pool sizes is needed to resolve the question. Nevertheless, the finding that BNIT does not increase bilirubin production, coupled with the inability of this agent to exert a hyperbilirubinemic response, tends to support the hypothesis that ANIT-induced enhancement of bilirubin production may play a role in the overall hyperbilirubinemic response caused by ANIT.

The mechanism by which the inhibitors of protein synthesis are diminishing ANIT-induced responses is not clear. The data presented indicate that the inhibitors, when given alone, do not depress incorporation of ALA-^{14}C into bilirubin. The results obtained with cycloheximide confirm those reported by Levitt *et al.*[18] These authors showed that the formation of nonerythropoietically derived bilirubin proceeded at normal rates or greater in rats treated with cycloheximide 1 hr before the administration of ALA-^{14}C, although protein synthesis was markedly reduced. They further showed that cycloheximide did not alter bilirubin excretion. There are several findings which implicate that the biotransformation of ANIT is involved in its hepatotoxic response.[19–23] Cytochrome P-450 concentrations have been shown to be markedly depressed 1 hr after cycloheximide administration.[18] However, it is not known whether the biotransformation of ANIT utilizes this pathway. Alterations in ANIT absorption must also be considered. Clearly, the elucidation of the mechanism of the protective effect of these agents should be attempted, for by such studies it might become possible to establish how ANIT exerts its own effects.

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