Isoniazid and Iproniazid: Activation of Metabolites to Toxic Intermediates in Man and Rat

Abstract. *Acetylhydrazine, a metabolite of isoniazid, a widely used antituberculosis drug, and isopropylhydrazine, a metabolite of iproniazid, an antidepressant removed from clinical use because of high incidence of liver injury, were oxidized by cytochrome P-450 enzymes in human and rat liver microsomes to highly reactive acylating and alkylating agents. Covalent binding of these metabolites to liver macromolecules paralleled hepatic cellular necrosis. The metabolites formed from these and probably other monosubstituted hydrazines are reactive electrophiles.*

In three recent clinical studies (*1*) we have found evidence that the hydrazine moiety of the widely used antituberculosis drug, isoniazid, may be responsible for the serious hepatitis that has led to curtailment of the use of the drug (*2*). The conversion of isoniazid to its principal metabolite, acetylisoniazid, is under genetic control in man (*3*). Examination of the metabolism of isoniazid and acetylisoniazid in human volunteers, with identification of metabolites by chromatography, reverse isotope dilution, and mass spectral analysis, showed that people having a fast acetylation phenotype are exposed to much more acetylisoniazid and acetylhydrazine than are people having a slow acetylation phenotype (*1*). In experimental animals, acylation of hepatic macromolecules and acute hepatic necrosis occurred after the administration of [^{14}C]acetylisoniazid and [^{14}C]acetylhydrazine (*4*). However, no covalent binding occurred after acetylisoniazid was administered when the ^{14}C was in the pyridine ring, indicating that the reactive metabolite arose only from the acetylhydrazine moiety. When the hydrolysis of acetylisoniazid to acetylhydrazine and isonicotinic acid was inhibited by administration of bis-*p*-nitrophenylphosphate, both necrosis and acylation were markedly decreased. The necrosis and acylation were potentiated by prior treatment of animals with phenobarbital and prevented by treatment with inhibitors of drug metabolism such as cobalt chloride and aminotriazole, even though phenobarbital, cobalt chloride, and aminotriazole do not affect the hydrolysis of acetylisoniazid. We, therefore, concluded that the hepatic necrosis and covalent binding were produced by further oxidative metabolism of the isoniazid metabolite, acetylhydrazine.

Studies in animals of the metabolism of iproniazid (isopropylisoniazid), an antidepressant drug removed from clinical use because of a high incidence of isoniazid-like hepatic injury (*5*), revealed that iproniazid also required enzymatic hydrolysis to produce the hepatic lesion. Specific labeling with ^{3}H and ^{14}C and studies of covalent binding showed that isopropylhydrazine was released by hydrolysis and further oxidatively activated in vivo to a potent hepatotoxin (*4*). The similar fates of acetylisoniazid and isopropylisoniazid support the view that the hepatic injury caused by hydrazide drugs is due to the metabolic activation of their hydrazine moieties.

For these reasons we have attempted to define the chemical reaction mechanisms by which acetylhydrazine, isopropylhydrazine, and other hydrazines that are presumably toxic are activated, and to determine whether this activation system is present in human tissues. Based on results in vivo with microsomal inducers and inhibitors, we conducted experiments in vitro with rat liver microsomes. The results of these experiments (Table 1) showed that (i) a reactive metabolite is formed which covalently binds to the microsomes, and (ii) the binding requires both reduced nicotinamide adenine dinucleotide phosphate (NADPH) and oxygen. Furthermore, experiments with hepatic microsomes prepared immediately following the traumatic death of a healthy young adult male demonstrate that the activation system is present in human tissues.

Table 1. Covalent binding in vitro of acetyl-[^{14}C]hydrazine and isopropyl[2-^{3}H]hydrazine to Fischer rat and human liver microsomes. Microsomes were prepared from rat liver and human liver and incubated as previously described (*15*). Each 3-ml sample contained 6 mg of rat microsomal protein (0.82 nmole of cytochrome P-450 per milligram of protein) or 3 mg of human microsomal protein (0.51 nmole of cytochrome P-450 per milligram of protein). Samples were incubated in air for 15 minutes with shaking (Dubnoff shaker incubator) at 37°C and covalent binding was determined (*15*). The values are means ± standard error (S.E.) of at least four determinations.

Conditions	Binding [nmole mg^{-1} (15 min)$^{-1}$]	
	1 m*M* Acetyl[^{14}C]-hydrazine	0.1 m*M* Isopropyl-[2-^{3}H]-hydrazine
Fischer rat microsomes		
Control	0.55 ± .052	0.58 ± .051
Minus NADPH	0.03 ± .011	0.06 ± .018
Atmosphere of 100 percent N_2	0.08 ± .009	0.06 ± .010
Gluthathione (1 m*M*)	0.19 ± .010	0.33 ± .025
Cysteine (1 m*M*)	0.27 ± .012	0.37 ± .029
Human microsomes		
Control	0.16 ± .020	0.37 ± .031
Minus NADPH	0.02 ± .005	0.03 ± .004

The electrophilic nature of the reactive species was suggested by experiments in which glutathione and cysteine were used to trap the toxic intermediates formed in the metabolism of acetyl- and isopropylhydrazine. Both of these sulfhydryl-containing compounds decreased the covalent binding to microsomes (Table 1). Glutathione and cysteine adducts, formed from the electrophilic intermediates arising from the metabolism of acetyl- and isopropylhydrazine, have been detected by thin-layer chromatography on Avicel F developed in *n*-propanol and water (70:30 by volume; R_F values of the glutathione adducts were 0.5 to 0.7). These adducts were not formed when either the NADPH-generating system or the sulfhydryl reagents were omitted from the reaction mixture.

Two of the adducts have been characterized. The glutathione conjugate formed in incubation mixtures containing acetylhydrazine was *S*-acetylglutathione as determined by recrystallization of the adduct to constant specific activity from 50 percent aqueous ethanol with synthetic *S*-acetylglutathione. The same procedure showed that *N*-acetylcysteine was formed in incubation mixtures containing the enzyme, NADPH, acetylhydrazine, and cysteine. The work of Smith and Gorin (*6*), which showed that *S*-acetylcysteine rearranges rapidly at neutral *p*H values to *N*-acetylcysteine, suggests that the initial product might have been *S*-acetylcysteine which subsequently rearranged to the observed product, *N*-acetylcysteine.

In order to study the activation process for acetylhydrazine in more detail, we administered mixtures of [^{14}C]-carbonyl- and [^{3}H]methyl-labeled acetylhydrazine (specific activity of ^{14}C, 0.36 mc/mmole, and of ^{3}H, 1.01 mc/mmole) to male Fischer rats (20 mg/kg). We used the same procedure in vitro with rat liver microsomes. These experiments (Table 2) showed that both carbon atoms of the acetyl group were bound because equivalent amounts of ^{14}C and ^{3}H were bound.

We conducted additional trapping experiments with cysteine and approximately equimolar amounts of acetylhydrazine and [methyl-$^{2}H_3$]acetylhydrazine. The *N*-acetylcysteine was isolated and chemical ionization mass spectrometry was performed (*7*). The

quasimolecular ions ($M+1$) at *m/e* 164 and 167 (VG Micro Mass 16F; accelerating voltage, 4 kv; electron energy, 100 ev; ionization current, 200 μa; isobutane reactant gas pressure ~ 0.3 torr) for the nonlabeled- and *N*-[methyl-$^2H_3$]-acetylcysteine were monitored and found to have the same H/^{2}H ratio as the quasimolecular ions at *m/e* 75 and 78 of the acetylhydrazine substrate mixture. These findings indicated that the entire acetyl group was bound and that the reactive intermediate was not ketene.

In addition to the acetylhydrazine experiments, mixtures of specifically labeled isopropylhydrazine (20 mg/kg; isopropyl[2-^{14}C]hydrazine, specific activity, 0.30 mc/mmole; isopropyl[2-^{3}H]-hydrazine, 0.87 mc/mmole) were administered to male rats and used as substrates in microsomal incubations. As is shown in Table 2, equivalent amounts of ^{3}H- and ^{14}C-labeled isopropylhydrazine were covalently bound to tissue macromolecules demonstrating that the C-2 methine hydrogen was retained, thus eliminating acetone as the intermediate for the binding reaction.

The formation of reactive metabolite from the microsomal oxidation of ^{3}H- and ^{14}C-labeled isopropylhydrazine was also followed by evolution of propane, which is similar to the production of methane by oxidative metabolism of methylhydrazine (*8*). Propane was trapped in septum-sealed vessels and determined by gas chromatography on a Perkin-Elmer 900 series gas chromatograph (Poropak Q, column temperature, 150°C; injector, 80°C; flame ionization detector, 200°C; N_2 carrier gas flow, 35 ml per minute; retention time of propane, 1.5 minutes). The evolution of propane was confirmed by combined gas chromatography and mass spectrometry, which showed the expected molecular ion at an *m/e* of 44 and fragment ions identical to those observed with authentic propane (LKB 9000*S*, 70 ev, 3.5 kv, 50 μa). The propane collected from incubation mixtures of isopropyl-[2-^{14}C]hydrazine and isopropyl[2-^{3}H]-hydrazine showed a ^{3}H/^{14}C ratio of 0.96 when compared to the ^{3}H/^{14}C ratio of the substrate mixture. This demonstrated that the propane arising from isopropylhydrazine retained the methine hydrogen. Moreover, the formation of propane paralleled the extent of the covalent binding that occurred when the rats were injected with phenobarbital or with phenobarbital plus cobalt chloride (see Table 3). Phenobarbital, which increased the activity of cytochrome P-450, increased the formation of propane and the covalent binding of metabolite, nearly doubling the latter. It is likely that some of the propane formed in vitro was further oxidized by the microsomes. In contrast, treatment of the animals with cobalt chloride plus phenobarbital decreased cytochrome P-450, covalent binding, and propane formation.

Table 2. Ratios of ^{3}H to ^{14}C bound to hepatic protein compared with the ratios in the initial substrate mixtures. Samples were prepared as in Table 1 and incubated with [^{14}C]acetylhydrazine and [^{3}H]acetylhydrazine or isopropyl[2-^{14}C]hydrazine and isopropyl[2-^{3}H]hydrazine as described in the text. Covalent binding of ^{3}H and ^{14}C to microsomal protein was determined as described (*15*) and found to be 0.15 nmole/mg (in vivo, acetylhydrazine), 0.49 nmole/mg per 15 minutes (in vitro, acetylhydrazine), 0.35 nmole/mg (in vivo, isopropylhydrazine), and 0.55 nmole/mg per 15 minutes (in vitro, isopropylhydrazine). Values are reported as ^{3}H/^{14}C ratios of the covalently bound metabolite, as determined by the channels-ratio method with integral counting (*16*), divided by the ^{3}H/^{14}C ratio of the initial substrate mixture as determined by the same method. Results are expressed as means ± S.E. of four such determinations.

Conditions	Substrate	
	Acetylhydrazine	Isopropylhydrazine
In vivo*	0.92 ± .021	0.96 ± .060
In vitro†	0.94 ± .012	0.98 ± .022

*Six hours after intraperitoneal injection of a mixture of [^{14}C]carbonyl- and [^{3}H]methyl-labeled acetylhydrazine (20 mg/kg). †After 15 minutes of incubation with the same mixture (1 m*M*).

Table 3. Correlation of propane evolution with covalent binding of isopropyl[2-^{3}H]-hydrazine to hepatic microsomes in vitro. Samples were incubated and covalent binding determinations were made as described in Table 1, except that microsomes were isolated from rats given intraperitoneal injections of phenobarbital (75 mg/kg) once a day for 4 days, or from rats given these injections for 4 days plus subcutaneous injections of cobalt chloride (30 mg/kg) once every 12 hours for a total of 48 hours. All incubation vessels were septum-sealed. The head-space gases were analyzed by gas-liquid chromatography as described in the text; the propane effluent was trapped in Aquasol scintillant cooled in a mixture of Dry Ice and acetone, and radioactivity was counted by scintillation spectrometry. Results are expressed as means ± S.E.

Treatment	Covalent binding [nmole (mg protein)$^{-1}$ (15 min)$^{-1}$]	Propane evolved (percent of total radioactivity in 15 min)
None	0.58 ± .051*	13.0 ± 1.00*
Phenobarbital	1.06 ± .059†	19.5 ± 0.75†
Phenobarbital and cobalt chloride	0.33 ± .006†	9.4 ± 0.81†

*Nine experimental determinations. †Six experimental determinations.

From our results we propose that many monoacyl and monoalkyl hydrazines are oxidized by cytochrome P-450 to *N*-hydroxy intermediates which then undergo dehydration to the respective diazenes. Although diazenes could be the reactive species, monosubstituted diazenes fragment in the presence of oxygen, most likely to radicals (*9*). Ketene has been eliminated as the reactive acylating species of acetylhydrazine by the study with [methyl-$^2H_3$]acetylhydrazine, which indicated that the entire acetyl group was bound. The studies with isopropylhydrazine, showing that changes in the rate of propane formation paralleled changes in the rate of binding of an isopropylhydrazine metabolite to tissue macromolecules with retention of the methine hydrogen in both metabolic pathways, suggests to us that the propane evolved and the metabolite which becomes covalently bound derive from a common intermediate. A reaction scheme which is consistent with the experimental results is the following:

$$R{-}\overset{H}{N}{-}NH_2 \rightarrow \xrightarrow[\text{oxidation}]{\text{Microsomal}} R{-}\overset{H}{N}{-}\overset{OH}{NH}$$

$$R{-}N{=}NH \rightarrow \xrightarrow[\text{or alkylation}]{\text{Tissue acylation}} R^+,\ R^{\cdot} \rightarrow RH$$

$$R = CH_3{-}C{=}O,\ CH_3{-}CH{-}CH_3$$

Another possible mechanism involves a second oxidation of the diazene to form a diazohydroxide, a reactive intermediate similar to that envisioned by Magee and Barnes (*10*) for carcinogenic nitrosamines and by Druckrey (*11*) for 1,2-dialkylhydrazines. However, we do not favor formation of a diazohydroxide because this intermediate does not produce alkanes (*8*). Thus, the reaction mechanism for reactive metabolite formation from monosubstituted hydrazines appears to be somewhat different from that for disubstituted hydrazines.

Whatever the intermediate may be, it is clear that oxidative activation of these hydrazines by microsomal enzymes mediates liver necrosis in animals. Since these enzymes are present in human liver tissue, these intermediates probably cause the serious and occasionally lethal hepatitis seen with isoniazid and iproniazid therapy in man.

Substituted hydrazine derivatives are receiving considerable attention in pharmacology and toxicology because of their widespread use as herbicides and rocket fuels, as intermediates in chem-

ical synthesis, and as therapeutic agents for the treatment of tuberculosis, depression, and cancer. Besides the liver necrosis found in therapy with isoniazid and iproniazid (*4*), hydrazines are known to produce many other toxic responses including methemoglobinemia, hemolysis, fatty liver, mutagenesis, and carcinogenesis (*12*). Although no evidence has yet been found that isoniazid is carcinogenic in man (*13*), the possible risk of neoplasia after long-term therapy with such drugs needs continual monitoring. The possibility that certain other hydrazine drugs such as carbidopa and phenelzine might be hepatotoxic in man should also be considered, because these hydrazines are apparently metabolized in part by mechanisms similar to those described for acetylhydrazine and isopropylhydrazine (*14*).

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Cholesterogenesis: Derepression in Extrahepatic Tissues with 4-Aminopyrazolo[3,4-*d*]pyrimidine

Abstract. *Administration of 4-aminopyrazolo[3,4-*d*]pyrimidine decreased serum cholesterol levels in the rat to less than 5 milligrams per deciliter. Coincident with this change, there was a 2.1- to 16.0-fold increase in the rate of sterol synthesis in seven extrahepatic tissues. This suggests that cholesterol carried in serum lipoproteins plays a major role in regulating sterol synthesis in many extrahepatic tissues.*

While all mammalian tissues that have been tested synthesize sterol in vitro, the highest rates of synthesis are usually found in liver and small intestine (*1*). Studies in vivo show these same two organs to be the major, if not the only, sources for circulating serum cholesterol (*2*). The rate of sterol synthesis in these tissues is responsive to a variety of control mechanisms: cholesterogenesis in the liver, for example, is modulated by such factors as stress, fasting, light-cycling, and variation in the amount of bile acid present in the animal (*3*). In addition, the rate of hepatic cholesterol synthesis varies inversely with the cholesterol content of the diet. This last control mechanism is mediated through the selective uptake by the liver of cholesterol carried in remnants of intestinal lipoproteins, and provides an important means for coupling the rate of endogenous sterol synthesis to the rate of intestinal absorption of exogenous, dietary cholesterol (*4*). Similarly, the rate of cholesterogenesis in the intestine also is responsive to such manipulations as fasting and alterations in the enterohepatic circulation of bile acids (*5*).

In contrast, sterol synthesis in essentially all other tissues in animals such as the rat and nonhuman primate occurs at very low rates and is not altered by such variables as light-cycling, fasting, bile acid pool size, or dietary cholesterol intake. However, in all of these situations the concentrations of lipoprotein-cholesterol in the serum are relatively constant. In view of the recent work in tissue culture showing that cholesterol carried in low density lipoproteins suppresses cholesterogenesis in the fibroblast (*6*), the possibility exists that sterol synthesis in one or more of the extrahepatic tissues also is suppressed by one of the major serum lipoprotein fractions.

To examine this possibility, we took advantage of the observation of Henderson and of Shiff *et al.* (*7*) that administration of the adenine analog 4-aminopyrazolo[3,4-*d*]pyrimidine (APP) apparently inhibits hepatic secretion of lipoproteins and causes a marked decrease in serum cholesterol levels. Using this analog, we have shown that cholesterol synthesis increases in at least seven extrahepatic tissues when the concentration of cholesterol in the serum is suppressed below 5 mg dl^{-1}.

Female, Sprague-Dawley rats initially weighing 190 to 210 g were subjected to light-cycling (12 hours of darkness and 12 hours of light) for 2 weeks. The light phase of the cycle began at 1500 hours while the dark phase began at 0300 hours. The animals were placed in individual metabolic cages and injected with either 0.9 percent NaCl solution (controls) or APP dissolved in 0.9 percent NaCl solution at a concentration of 5 mg ml^{-1} (experimental animals). The exact schedule for the injections is given in the legends to Table 1 and Fig. 1. During this treatment period each control animal was pair-fed with an animal receiving APP. To dissolve the APP (Sigma Chemical) it was necessary to titrate the solution to a *p*H of approximately 1.5 to 2.0 and then back-titrate to a *p*H of 4.0 just before giving the intraperitoneal injections. At the completion of the treatment period the animals were killed at 0900 hours, the mid-dark point of the light cycle, and various tissues were removed and immediately chilled in cold 0.9 percent NaCl solution. Tissue slices 0.8 mm thick were prepared and 300-mg (wet weight) portions were placed in 25-ml Erlenmeyer flasks containing 2.0 ml of oxygenated Krebs bicarbonate buffer (*p*H 7.4), 16 μmole of sodium acetate, and 10 mc of ^3H_2O. One set of flasks was incubated for 90 minutes at 37°C in a metabolic shaker set at 120 cycles per minute. A duplicate set of flasks was packed in ice and incubated with shaking under identical conditions at 0°C. At the end of the incubation period, 6 ml of alcoholic potassium hydroxide solution was added to each flask, and the tissues were saponified on a steam bath. Sterols were extracted with petroleum ether and precipitated as the digitonide. The radioactivity in the digitonin-precipitable sterols from the flasks incubated at 0°C was subtracted from the radioactivity in the digitonin-precipitable sterols obtained in the corresponding tissues incubated at 37°C. This difference was used to calculate the nanomoles of ^3H_2O incorporated into dig-