Biochemical Pharmacology, Vol. 30, No. 12, pp. 1651–1658, 1981.
Printed in Great Britain.

0006-2952/81/121652-08 $02.00/0

MECHANISM OF THE INHIBITORY ACTION OF ISONIAZID ON MICROSOMAL DRUG METABOLISM

SAMAR F. MUAKKASSAH, WAYNE R. BIDLACK and WILLIAM C. T. YANG*
Department of Pharmacology and Nutrition, University of Southern California School of Medicine, Los Angeles, CA 90033, U.S.A.

(*Received* 5 *August* 1980; *accepted* 12 *December* 1980)

Abstract—Addition of isoniazid (isonicotinic acid hydrazide, INH) to rat liver microsomes produced an immediate decrease in the binding of carbon monoxide to reduced cytochrome P-450. Preincubation of the microsomes with INH in the presence of NADPH produced a further decrease of carbon monoxide binding to cytochrome P-450. The latter decrease of functional cytochrome P-450 was dependent upon NADPH and oxygen and was transitory. Examination of compounds structurally related to INH indicated that both the hydrazine moiety and the aromatic ring were needed to produce both effects. Incubation of microsomes with INH also resulted in gradual increases in absorbance at 449 nm and at 493 nm which also were transitory. Thus, the decreased binding of carbon monoxide to cytochrome P-450 may have occurred concurrently with formation of these spectral intermediates. Microsomal *N*-demethylation and aniline *p*-hydroxylation were inhibited by isoniazid. Preincubation of the microsomes with INH and NADPH increased the inhibition. Thus, the decreased availability of cytochrome P-450 as observed may account for the inhibition of the mixed function oxidases by isoniazid.

Isoniazid (isonicotinic acid hydrazide INH) is a first line drug for the treatment of tuberculosis [1]. The major route of metabolism of isoniazid is acetylation and subsequent hydrolysis to isonicotinic acid and acetylhydrazine [2, 3]; the rate of acetylation in the population is genetically determined [4].

Although direct metabolism of isoniazid by the microsomal mixed function oxidases has not been demonstrated, isoniazid has been reported to interfere with this system. Patients who received diphenylhydantoin (DPH) as anticonvulsant therapy showed toxic symptoms in response to DPH when isoniazid was added to the treatment regimen, especially in those who were slow acetylators [5, 6]. Kutt *et al.* [7] showed that isoniazid, by inhibiting the metabolism of DPH, can increase DPH plasma concentration, resulting in toxic manifestations in these patients. The inhibition of the parahydroxylation of DPH *in vivo* by isoniazid has also been confirmed in cats and rats [7, 8]. In another clinical situation, isoniazid has been reported to inhibit the metabolism of primidone [9]. Additional *in vivo* observations that suggest that isoniazid may interfere with microsomal mixed function oxidase activity include prolongation of prothrombin time and hemorrhage associated with warfarin therapy in a patient taking isoniazid [10], enhancement of bishydroxycoumarin activity and elevation of prothrombin time in the dog after concomitant administration of isoniazid [11] and prolongation of methohexital sleeping time and zoxazolamine paralysis time by isoniazid in rats [12].

* Author to whom all correspondence should be addressed: William C. T. Yang, Ph.D., Department of Pharmacology and Nutrition, University of Southern California School of Medicine, Health Sciences Campas, 2025 Zonal Ave., Los Angeles, CA 90033, U.S.A.

In addition, inhibition of drug metabolism by isoniazid *in vitro* has been reported, including the metabolism of diphenylhydantoin in rat liver 9000 *g* supernatant and microsomal fractions [7, 13], the metabolism of pentobarbital, carisoprodol and aminopyrine in rat liver 9000 *g* supernatant fraction [14] and, more recently, the microsomal metabolism of acetylhydrazine in rat liver [15]. The mechanism of inhibition of liver microsomal drug metabolism by isoniazid, however, remains unknown.

The present study was designed to investigate the mechanism of the inhibitory action of isoniazid on microsomal drug metabolism in the liver. We report here that isoniazid caused a detectable decrease in carbon monoxide binding to reduced cytochrome P-450, and suggest that this decreased availability of functional cytochrome P-450 may account for the observed inhibition of microsomal mixed function oxidase activity.

METHODS

Chemicals. Isoniazid, nicotinic acid hydrazide, iproniazid phosphate, aniline and aminopyrine were purchased from the Aldrich Chemical Co., Inc., Milwaukee, WI. *N*-Acetylhydrazine, isonicotinamide and benzoylhydrazine were purchased from I.C.N. Pharmaceuticals Inc. Life Science Group, Plainview, NY. The *N*-oxide of isoniaxid was a gift from David Srulevitch, USC, Los Angeles, CA. Sodium phenobarbital was supplied by the J. T. Baker Chemical Co., Phillipsburg, NJ, and 3-methylcholanthrene was purchased from the Eastman Kodak Co., Rochester, NY.

Animal treatment. Male Sprague–Dawley rats weighing 200–250 g were used. All animals had free access to food and water. The phenobarbital-treated rats were given phenobarbital (0.15%) in their drink-

ing water and were allowed to drink *ad lib.* for 5 days. The 3-methylcholanthrene (3-MC)-treated rats were injected i.p. with 3-MC in corn oil (20 mg kg^{-1} day^{-1}) i.p. for 2 days and were killed on day 4.

Preparation of hepatic microsomes. Freshly prepared rat liver microsomes were used in all experiments. Microsomes were isolated as described previously [16]. For the spectral studies, EDTA (0.1 mM) was included in the washing medium to minimize the endogenous lipid peroxidation activity initiated by NADPH in the microsomes during incubation [17]. Protein was determined by the method of Lowry *et al.* [18].

Cytochrome P-450 determination. Hepatic microsomes were suspended (1 mg protein/ml) in 50 mM Tris–Cl buffer solution (pH 7.4) containing 150 mM KCl and 10 mM $MgCl_2$. Various amounts of NADPH and isoniazid or related compounds were added to the incubation mixture as indicated. The microsomal suspension was either incubated for various periods of time or used immediately for cytochrome P-450 determinations. Incubations were carried out in a temperature-regulated water bath at 37°. At the end of the reaction the tube containing the microsomal suspension was transferred to ice, and the suspension was reduced immediately with sodium dithionite and divided between two cuvettes. The contents of the sample cuvette were gassed for 90 sec with carbon monoxide and the difference spectrum was then recorded. Cytochrome P-450 content was determined by difference spectrophotometry according to the method of Omura and Sato [19], using an extinction coefficient between 450 and 490 nm of 91 $mM^{-1}cm^{-1}$. For incubations performed under anaerobic conditions, the ice-cold reaction buffer was bubbled with argon for 3 min and sealed under an argon atmosphere prior to the addition of the microsomes. The microsomes were kept on ice and were also gently flushed with a stream of argon. Aliquots of microsomes were removed from this flask with a syringe and injected into the reaction tubes to initiate the reaction.

Spectral studies. EDTA-washed microsomes, isolated from phenobarbital-treated rats, were suspended (1 mg protein/ml) in the standard buffer. The suspension was reduced with NADH (220 μM) and divided between two cuvettes, and a flat baseline was established. After the addition of isoniazid (1 mM) to the sample cuvette and of an equal volume of buffer to the reference cuvette, the difference spectrum was recorded. NADPH was then added to the sample cuvette to give a final concentration of 250 μM. The difference spectra were recorded after various periods of time. The spectrophotometric determinations were done at 37° using a temperature-regulated Aminco DW-2 spectrophotometer.

In vitro *drug metabolism.* Hepatic microsomes from phenobarbital-treated rats were suspended at either 0.75 mg/ml or 1 mg/ml in the buffer mentioned above for reactions involving, respectively, aminopyrine *N*-demethylation or aniline hydroxylation. The microsomes were preincubated at 37° for 3 min with either 0.1 or 1.0 mM isoniazid in the presence or absence of NADPH (125 μM). Drug metabolism was then initiated by the simultaneous addition of aminopyrine (10 mM) or aniline (1 mM) and NADPH (500 μM). The final volume of the reaction mixture was 1 ml. Metabolism of aminopyrine was terminated at 3 min, whereas metabolism of aniline was allowed to proceed for 15 min. The reactions were terminated by the addition of ice-cold trichloroacetic acid (0.5 ml of 20%). Protein was removed by centrifugation at 1500 *g* for 15 min at 4°. The supernatant fractions were assayed for formaldehyde using the method of Nash [20], or for *p*-aminophenol by the method of Imai *et al.* [21]. Under these conditions formation of products was linearly related to incubation time and to the amount of microsomal protein.

RESULTS

Effect of isoniazid on drug metabolism in vitro. Isoniazid (0.1 and 1.0 mM) inhibited aminopyrine *N*-demethylation and aniline hydroxylation in rat liver microsomes (Table 1). These results further demonstrate the ability of isoniazid to inhibit microsomal mixed function oxidase activity, as has been reported by Kutt *et al.* [7] for DPH. When NADPH was included with isoniazid in the preincubation of the microsomal suspension, the inhibition was even greater.

Table 1. Inhibition of the *in vitro* metabolism of aminopyrine and aniline by isoniazid*

Isoniazid (mM)	Inhibition of drug metabolism†,‡ (%)			
	Preincubation without NADPH		Preincubation with NADPH	
	Aniline	Aminopyrine	Aniline	Aminopyrine
0.1	8.3	17.5	20.0	27.1
1.0	29.0	30.0	53.5	54.0

* Control aminopyrine *N*-demethylase activities of microsomes isolated from the livers of phenobarbital-treated rats were 41.0 and 38.0 nmoles HCHO produced·min^{-1}·mg^{-1} for preincubations carried out in the absence or presence of NADPH respectively. Control aniline hydroxylase activities were respectively, 14.2 and 13.1 nmoles *p*-aminophenol produced·mg^{-1}·15 min^{-1}.

† Preincubation of the microsomal suspension for 3 min was carried out in the presence or absence of 125 μM NADPH. The reaction was then initiated by the simultaneous addition of 10 mM aminopyrine, or 1 mM aniline, and 500 μM NADPH.

‡ Each value is the average of two separate experiments performed in duplicate.

Effect of isoniazid on microsomal cytochrome P-450 in vitro. To investigate the mechanism by which isoniazid inhibits the hepatic metabolizing system, studies were designed to explore the possibility of a direct interaction with cytochrome P-450. A microsomal suspension containing NADPH was divided equally between a sample and a reference cuvette, providing a balanced flat baseline. Isoniazid was added to the sample cuvette and an equal amount of buffer to the reference cuvette. The contents of the sample and reference cuvettes were then either directly reduced with sodium dithionite or incubated for 7.5 min at 37° prior to the addition of sodium dithionite. Difference spectra with absorption maxima at 444 nm (Fig. 1a, 0 min) or at 447 nm (Fig. 1b, 7.5 min) were obtained. Since isoniazid itself produced an absorption maximum in the Soret region with dithionite reduced microsomes, cytochrome P-450 quantitation was carried out as described in Methods. Essentially, the microsomal suspension, after various treatments in the presence or absence of drug, was reduced with sodium dithionite and then divided between the sample and reference cuvettes. After a flat baseline was thus obtained, the cytochrome P-450 content was determined in the presence of CO.

The addition of isoniazid (0.1 to 2.0 mM) to hepatic microsomes prepared from phenobarbital-treated rats caused a decrease (3–20 per cent) in the cytochrome P-450 absorption spectrum as measured by CO binding to sodium dithionite reduced microsomes (Fig. 2). Further decreases in cytochrome P-450 (18–45 per cent) were produced by incubation

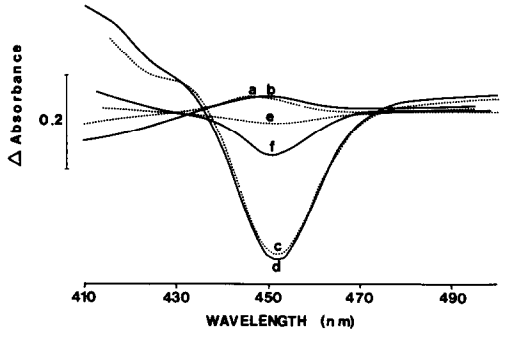

Fig. 1. Difference spectra produced by interaction of isoniazid with cytochrome P-450. Hepatic microsomes from phenobarbital-treated rats were suspended at 2 mg/ml in 50 mM Tris–Cl buffer solution containing 150 mM KCl and 10 mM $MgCl_2$ in the presence of NADPH (250 μM). The suspension was divided between two cuvettes. Isoniazid (1 mM) was added to the sample cuvette, and an equal volume of buffer to the reference cuvette. The contents of the sample and reference cuvettes were then either reduced immediately with sodium dithionite and the difference spectrum recorded (curve a) or incubated at 37° for 7.5 min prior to sodium dithionite addition (curve b). Tracings c and d were obtained under conditions similar to those of curves a and b, respectively, except that CO was bubbled in the reference cuvette for 90 sec. Tracings e and f were obtained as a and b were, respectively, except that CO was bubbled in both the sample and reference cuvettes for 90 sec.

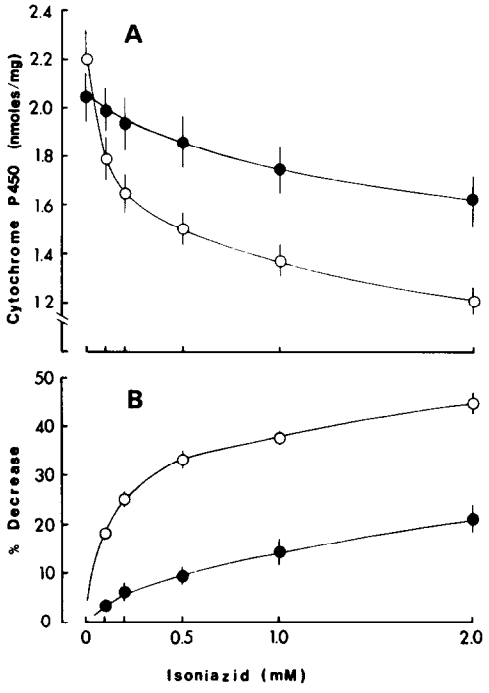

Fig. 2. Panel A: Effect of isoniazid (0.1 to 2.0 mM) on cytochrome P-450 of hepatic microsomes prepared from phenobarbital-treated rats. Key: (●) before incubations; (○) after incubation with isoniazid (7.5 min, 37°). Initial NADPH concentration was 250 μM. Each value is the mean ± S.E.M. of four separate experiments (●) and seven separate experiments (○) run in duplicate. Panel B: The results in Fig. 2A are expressed as per cent decrease of cytochrome P-450 compared to control values in the absence of isoniazid. Where not shown, the S.E.M. is less than the radius of the symbol.

(37°, 7.5 min) in the presence of NADPH. Similar results were also obtained from untreated rats and rats treated with 3-MC (Table 2). Multiple forms of cytochrome P-450 have been shown to exist in rat liver [22], some of which could be specifically induced and purified from phenobarbital (cytochrome P-450)- and 3-MC (cytochrome P-448)-treated rats [23]. The initial per cent decrease in cytochrome P-450, prior to the incubation of the microsomal suspension, and the further decrease caused by incubation with isoniazid were approximately the same in microsomes prepared from control, phenobarbital- or 3-MC-treated rats. These results suggest that the effect of isoniazid is not specific toward the specific forms of cytochrome P-450 (P-450 and P-448) that are inducible by phenobarbital and 3-MC respectively.

To eliminate the possibility of a decrease in measurable cytochrome P-450 due to endogenous CO generation, carbon monoxide was gassed into both the sample and reference cuvettes for 90 sec. This provided sufficient CO to bind all of the available cytochrome P-450 [19]. The resulting difference spectra showed a negative absorbance at 450 nm (Fig. 1, curves e and f), indicating that there was less cytochrome P-450 available for CO binding in the sample cuvette. This negative absorbance was

Table 2. Effect of isoniazid (INH) on hepatic microsomal cytochrome P-450 content *in vitro**

Condition	INH addition (mM)	Cytochrome P-450 (nmoles/mg)		
		Control	Phenobarbital-treated	3-Methylcholanthrene-treated
No incubation	0.0	0.97 ± 0.005†	2.04 ± 0.05	1.59 ± 0.03
	0.5	0.84 ± 0.005 (13)‡	1.78 ± 0.04 (12)	1.42 ± 0.04 (10)
	2.0	0.78 ± 0.005 (19)	1.61 ± 0.02 (21)	1.24 ± 0.07 (22)
With incubation§	0.0	1.07 ± 0.015	2.15 ± 0.05	1.64 ± 0.06
	0.5	0.78 ± 0.015 (27)	1.46 ± 0.03 (34)	1.17 ± 0.08 (29)
	2.0	0.66 ± 0.010 (38)	1.20 ± 0.02 (44)	0.80 ± 0.04 (51)

* For details of treatment, see Methods.

† Values are means ± S.E.M. of four experiments run in duplicate for the control group and three experiments for the other two groups.

‡ Values in parentheses show the per cent decrease compared to the control in the absence of isoniazid within the same group of animals.

§ Hepatic microsomes were incubated at 37° in the presence of 250 μM NADPH for 7.5 min and INH as indicated.

greater for curve f which was obtained after 7.5 min of incubation. Since both cuvettes were gassed with CO, the endogenously generated CO, if any, could not account for the observed decrease in cytochrome P-450. Curves c and d in Fig. 1 show the difference spectra at 0 and 7.5 min with only the reference cuvette gassed with CO, illustrating that the cytochrome P-450 content did not change after 7.5 min of incubation.

Effect of NADPH and oxygen on isoniazid-induced decrease of microsomal cytochrome P-450 in vitro. In the absence of NADPH or oxygen, INH still produced a similar initial decrease in CO binding to sodium dithionite reduced microsomes (Table 3), but no further decrease in cytochrome P-450 after incubation with isoniazid was observed. To determine whether this decrease was mediated by hydrogen peroxide generated in the microsomes, the effect of added exogenous catalase (290 units/ml) was evaluated; it was found to have no effect.

Comparison of the effects of isoniazid and related compounds on cytochrome P-450 in vitro. Experiments were performed to determine the molecular structures required for the observed effect of isoniazid on cytochrome P-450. Nicotinic acid hydrazide, an isomer of isoniazid, had the same effects on cytochrome P-450 (Table 4). The *N*-oxide of isoniazid had exerted similar effects but they were smaller. Iproniazid, which has been reported to inhibit drug metabolism [24, 25] and which contains an isopropyl substituent on the hydrazine group, caused a greater initial decrease than isoniazid but no further decrease occurred after incubation. Isonicotinamide had no effect. These results indicate the importance of the hydrazine moiety. In addition, benzoyl hydrazine had a greater effect than isoniazid, whereas acetyl hydrazine had no effect, suggesting that the aromatic moiety in isoniazid and related compounds also is important in this activity, due perhaps to its lipophilic character.

Effect of NADPH concentration and time of incubation on isoniazid-induced decrease of cytochrome P-450 in vitro. Loss of cytochrome P-450 was also noted when the microsomal suspension was incubated in the presence of NADPH lacking isoniazid for periods longer than 7.5 min (Fig. 3). This decrease has been noted previously [26, 27] and is related to peroxidation of the membrane lipids. During microsomal peroxidation, cytochrome P-450 and its heme moiety are degraded [27, 28]. In addition, CO is formed in part from the heme metabolism but to a greater extent from the peroxidizing lipids [16].

Table 3. Effect of NADPH and oxygen on isoniazid (INH) induced reduction of microsomal cytochrome P-450*

Condition	Additions	Cytochrome P-450 (nmoles/mg)	
		No incubation	With incubation†
Aerobic	None	2.05 ± 0.02‡	2.23 ± 0.08
	INH (1 mM)	1.80 ± 0.04 (12)§	2.02 ± 0.07 (9)
	NADPH (0.25 mM)	2.03 ± 0.02	2.06 ± 0.03
	NADPH (0.25 mM), INH (1 mM)	1.79 ± 0.04 (12)	1.27 ± 0.02 (38)
Anaerobic	None	2.27 ± 0.05	2.21 ± 0.05
	INH (1 mM)	2.04 ± 0.05 (10)	1.98 ± 0.09 (10)
	NADPH (0.25 mM)	2.18 ± 0.03	1.74 ± 0.09
	NADPH (0.25 mM), INH (1 mM)	1.97 ± 0.03 (10)	1.60 ± 0.07 (9)

* Microsomes were isolated from the livers of rats treated with phenobarbital.

† Incubation of the microsomal suspensions (1 mg/ml) was carried out at 37° for 7.5 min under the conditions and with the additions listed above.

‡ Each value is the mean ± S.E.M. of three experiments run in duplicate.

§ Values in parentheses show the per cent decrease compared to the control in the absence of isoniazid.

Table 4. Effects of isoniazid and related compounds on microsomal cytochrome P-450 contents *in vitro*

Drug (1 mM)	% Decrease in cytochrome P-450*	
	No incubation	With incubation†
Isoniazid	9 ± 2.0	32 ± 1.5
Isonicotinamide	7 ± 1.0	(3 ± 0.4)‡
Iproniazid	23 ± 2.0	20 ± 0.5
Acetyl hydrazine	1 ± 1.5	(5 ± 2.0)‡
Nicotinic acid hydrazide	7 ± 1.0	31 ± 2.0
N-Oxide of isoniazid	2 ± 1.5	11 ± 2.5
Benzoyl hydrazine	30	42
Metyrapone§	34 ± 2.5	21 ± 1.0

* Values show the per cent decrease compared to the control in the absence of the compound. Microsomes were isolated from the livers of rats treated with phenobarbital. Control cytochrome P-450 values were 2.17 ± 0.2 and 2.14 ± 0.2 with no incubation and with incubation respectively. Values for benzoyl hydrazine are the averages of two separate experiments. All the other values are expressed as the means ± S.E.M. of three separate experiments run in duplicate.

† Incubation of the microsomal suspension (1 mg/ml) was carried out at 37° for 7.5 min in the presence of 250 μM NADPH and oxygen.

‡ Values show the increase in assayable cytochrome P-450.

§ Metyrapone was included here only for comparison.

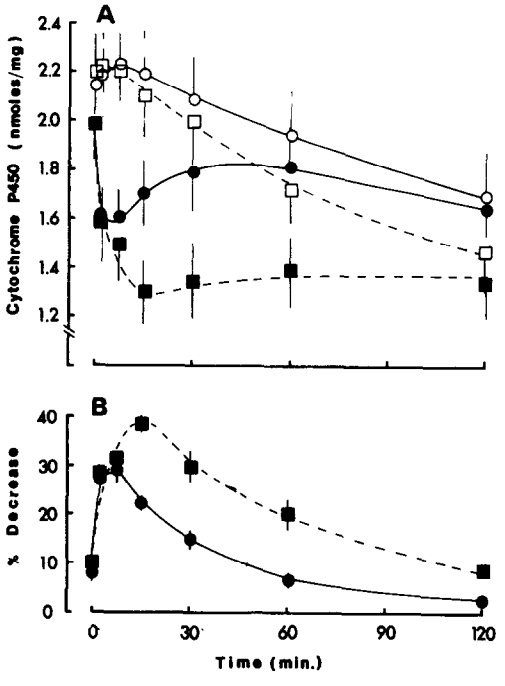

Fig. 3. Panel A: Effect of isoniazid (1 mM) on cytochrome P-450 of hepatic microsomes from phenobarbital-treated rats at various times after incubation in the absence of isoniazid (○, □) or in the presence of isoniazid (●, ■). NADPH concentration was either 125 μM (○, ●) or 250 μM (□, ■). Each value is the mean ± S.E.M. of three separate experiments run in duplicate. Panel B: The results of Fig. 3A are expressed as per cent decrease of cytochrome P-450 compared to control values in the absence of isoniazid. Where not shown, the S.E.M. is less than the radius of the symbol.

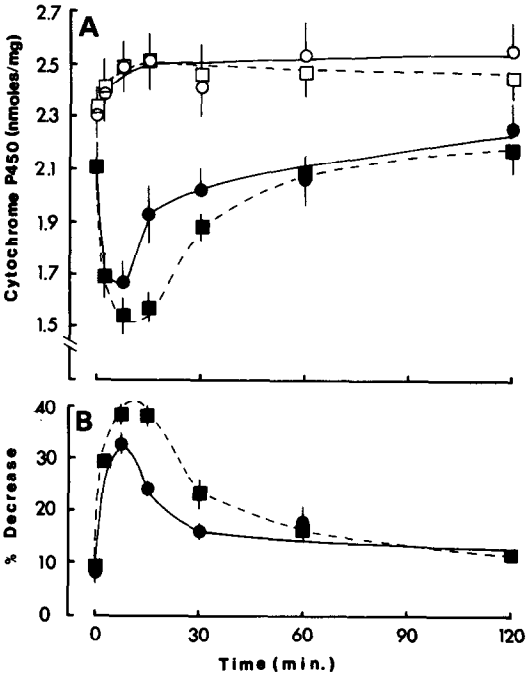

Fig. 4. Effect of 1 mM isoniazid in the presence of 1 mM GSH. Cytochrome P-450 of hepatic microsomes from phenobarbital-treated rats was measured at various times after incubation in the absence (○, □) or in the presence of isoniazid (●, ■). NADPH concentration was either 125 μM (○, ●) or 250 μM (□, ■). Each value is the mean ± S.E.M. of three separate experiments run in duplicate. Panel B: The results of Fig. 4A are expressed as a per cent decrease of cytochrome P-450 compared to control values in the absence of isoniazid. Where not shown, the S.E.M. is less than the radius of the symbol.

This background CO may have interfered with quantitation of the total cytochrome P-450 since the microsomal suspension lacking the drug in the reference cuvette was used to obtain the difference spectrum. Upon incubation of the microsomal suspension in the presence of NADPH (125 or 250 μM) and isoniazid (1 mM), a reversible decrease in cytochrome P-450 was observed; the maximum decrease was found after 7.5 min. Although the cytochrome P-450 loss could not be quantitated precisely, since the control microsomes lost cytochrome P-450 as well, the effect of isoniazid still appears to have been an increase in loss of cytochrome P-450 (Fig. 3). To prevent this gradual decrease of cytochrome P-450 with time, glutathione (1 mM) was added to the reaction system to reduce the background lipid peroxidation [29, 30]. As see in Fig. 4, inclusion of glutathione in the microsomal suspension was accompanied by a constant level of cytochrome P-450 throughout the entire incubation period. Incubation of the microsomal suspension with NADPH (125 μM), glutathione (1 mM), and isoniazid (1 mM) resulted also in a reversible decrease of cytochrome P-450 with time (Fig. 4). Increasing the NADPH concentration to 250 μM during incubation slightly increased the maximum effect and significantly delayed the onset of reversal to 15 min (Fig. 4). This transient decrease in cytochrome P-450 could be elicited again after complete reversal had occurred (1 hr) by the addition of more NADPH (Fig. 5). Thus, the transient nature of this effect *in vitro* may have been due to the decreased NADPH concentration during prolonged incubation. This effect may therefore be important *in vivo* since NADPH is generated continuously.

Spectral studies. As shown in Fig. 6, isoniazid bound to cytochrome P-450 to give a typical type II binding spectrum (curve a). In the presence of NADH in both sample and reference cuvettes, a similar binding spectrum was obtained (Fig. 6b). NADH was routinely added to the sample and reference cuvettes to eliminate the influence of reduced cytochrome b_5 on the difference spectral recordings. Curve c in Fig. 6 was similarly obtained with dithionite in both cuvettes. This is the same curve noted in Fig. 1a but it is plotted on an expanded O.D. scale. The spectral perturbations produced by the interaction of isoniazid with hepatic microsomes isolated from phenobarbital-treated rats are shown in Fig. 7. Upon the addition of isoniazid (1 mM) and NADPH (250 μM) to the sample cuvette, an absorbance maximum at 449 nm and a broad absorbance at 493 nm appeared, both of which increased with time. A minimum absorbance at 418 nm was observed as well. If followed for an extended period of time (30 min), the 449 nm peak reached a maximum by 15 min and then slowly decreased. The 449 nm peak was dependent upon oxygen and NADPH, but not on NADH. In addition, the absorbance at the 449 nm peak was dependent upon NADPH concentration, decreasing earlier (7–8 min) at a lower NADPH concentration (125 μM). The inclusion of 1 mM GSH and/or saturation of the buffer with oxygen did not produce changes in the gradual formation and decrease of the 449 nm absorbance maximum.

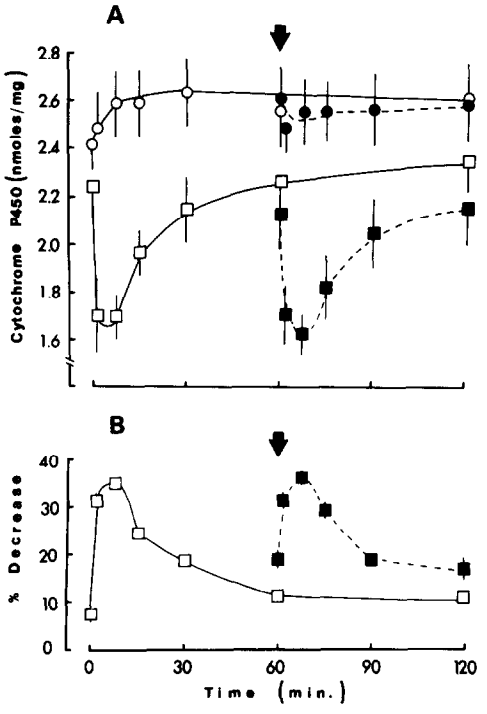

Fig. 5. Panel A: Cytochrome P-450 in hepatic microsomes from phenobarbital-treated rats incubated in the absence (○, ●) or the presence (□, ■) of 1 mM isoniazid. Initial concentration of NADPH was 125 μM. After 1 hr, NADPH (to increase the final concentration to 125 μM) was added and cytochrome P-450 was determined again (●, ■). GSH (1 mM) was included in all incubation tubes. Each value is the mean ± S.E.M. of three separate experiments performed in duplicate. Panel B. The results in Fig. 5A are expressed as per cent decrease of cytochrome P-450 compared to control values in the absence of isoniazid. Where not shown, the S.E.M. is less than the radius of the symbol.

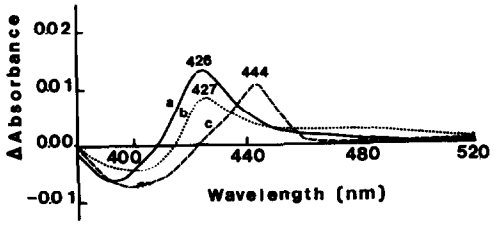

Fig. 6. Microsomal binding spectra of isoniazid. Hepatic microsomes from phenobarbital-treated rats (cytochrome P-450 = 1.7 nmoles/mg) were suspended at 1 mg/ml in 50 mM Tris–Cl buffer solution containing 150 mM KCl and 10 mM $MgCl_2$. The suspension was either used as it was or reduced with either NADH (250 μM) or a few grains of sodium dithionite. The suspension was then divided between two cuvettes and a flat baseline with equal light absorbance was established. Isoniazid (1 mM) was added to the sample cuvette and an equal volume of buffer was added to the reference cuvette, and the difference spectrum was recorded. Curves a, b and c show, respectively, the binding spectra of isoniazid to oxidized microsomes, NADH reduced microsomes, and dithionite reduced microsomes.

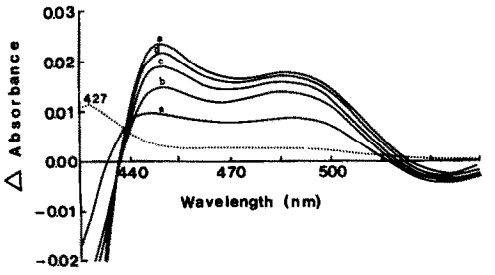

Fig. 7. Microsomal spectral changes produced by isoniazid. Hepatic microsomes (1 mg/ml) from phenobarbital-treated rats (cytochrome P-450 = 2.9 nmoles/mg) were used. The spectral changes were recorded as described in Methods. The dotted line represents a portion of the drug binding spectrum obtained after the addition of isoniazid to the NADH reduced microsomes. Tracings a, b, c, d, and e represent, respectively, the difference spectra recorded 20 sec, 2.5 min, 5 min, 7.5 min and 10 min, after the addition of NADPH (250 μM) to the sample cuvette.

DISCUSSION

We reported here that isoniazid inhibits the microsomal metabolism of both aminopyrine *N*-demethylation and aniline *p*-hydroxylation (Table 1). This confirms the ability of isoniazid to inhibit microsomal mixed function oxidase activity as was reported previously [7, 14]. Kutt *et al.* [7] reported that 0.1 and 1.0 mM isoniazid inhibited the microsomal metabolism of DPH by 46 and 100 per cent respectively, whereas our results showed a maximum inhibition of 20 and 50 per cent at 0.1 and 1.0 mM isoniazid respectively. The greater potency of isoniazid inhibition of DPH metabolism may be due to a limited capacity of microsomal enzyme(s) to hydroxylate DPH [31, 32].

Our finding that isoniazid produced a decrease in carbon monoxide binding to reduced cytochrome P-450 and, by analogy, a decrease in oxygen binding to cytochrome P-450 suggests that this may be, in part, the mechanism by which isoniazid inhibits cytochrome P-450-dependent oxidations. The decrease in carbon monoxide detectable cytochrome P-450 caused by isoniazid had two phases, an initial decrease before incubation and a further decrease after incubation of the microsomes in the presence of NADPH and oxygen (Fig. 2). The initial decrease may have been due to direct interference of isoniazid with the binding of CO to cytochrome P-450. When added directly to sodium dithionite reduced microsomes, isoniazid exhibited a weak binding spectrum with an absorbance maximum at 444 nm and an absorbance minimum at 395 nm, indicating a ligand interaction with the ferrous heme of cytochrome P-450 (Fig. 1a). Other inhibitors, such as metyrapone, which bind to the ferrous heme of cytochrome P-450 also have been reported to decrease carbon monoxide binding to reduced cytochrome P-450 [33]. Metyrapone and iproniazid produced a more profound difference spectrum than isoniazid, with an absorbance maximum at 446 nm, when added to dithionite reduced microsomes.* Under our conditions, they also decreased to a greater extent the binding of CO to reduced cytochrome P-450 (Table 4). Unlike isoniazid, however, neither metyrapone nor iproniazid produced any further decrease in cytochrome P-450 upon preincubation with the microsomes in the presence of oxygen and NADPH. As shown in Table 3, the decrease in cytochrome P-450 after incubation did not occur in the absence of either NADPH or oxygen (conditions necessary for mixed function oxidations to occur). Furthermore, incubation of the liver microsomes with isoniazid required the presence of NADPH and oxygen to give rise to the formation of an absorbance maximum at 449 nm (Fig. 7). Moreover, the presence of NADPH and oxygen during the incubation resulted in greater inhibition of aminopyrine *N*-demethylation and aniline *p*-hydroxylation (Table 1).

In recent years it has been noted that the oxidative conversion of certain inhibitors, via cytochrome P-450 catalyzed reactions, results in the formation of a reduced cytochrome P-450 metabolic intermediate complex (MI) that is readily identifiable spectrophotometrically. These inhibitors include methylenedioxybenzene derivatives such as safrole [34, 35] and piperonyl butoxide [36–39] and nitrogenous amines such as SKF-525A [40, 41] and amphetamine derivatives [42–45]. Franklin [46] extended the list to include *p*-chloroaniline, sulfanilamide, 2-methylindole and dapsone. The formation of these metabolic intermediate complexes requires NADPH and oxygen; the complexes exhibit an absorbance maximum in the Soret region between 448 and 456 nm when the heme iron is in the reduced ferrous state. The metabolic intermediate complex is not displaced by carbon monoxide, thus the content of functional cytochrome P-450 decreases, resulting in the inhibition of the mixed function oxidations [47].

Although our results are similar in certain respects to these published results, there are differences. First, in our spectral studies (Fig. 7) a broad absorbance at 493 nm was present. None of the above mentioned MI complexes has been reported to absorb in this region. Second, isoniazid is structurally unrelated to the MI complex-forming inhibitors reported. Third, no direct metabolism of isoniazid by the microsomal mixed function oxidases has been reported. Our finding of a time-dependent formation of a complex of isoniazid with cytochrome P-450 suggests that direct metabolism of isoniazid by the microsomal oxidases is a possibility that may be especially important in patients who acetylate isoniazid slowly. Prough and co-workers [48–50] have presented evidence for the involvement of the liver microsomal amine oxidase and a cytochrome P-450-dependent enzyme system in the metabolism of some hydrazine derivatives. Our results suggest the importance of the hydrazine moiety of isoniazid (Table 4). Under similar conditions, isonicotinamide, which has an amide instead of a hydrazide group, neither decreased cytochrome P-450 nor caused the formation of a metabolic intermediate complex in liver microsomes. In addition, isonicotinamide did not inhibit either aniline hydroxylation or aminopyrine *N*-demethylation to any appreciable extent.* Fourth, the transient nature of the metabolic inter-

* Unpublished observations.

mediate complex and the reversibility of the decrease in cytochrome P-450 by isoniazid *in vitro* is distinctly different from the more stable form of the complexes observed by Franklin and others [47, 51]. In spite of its *in vitro* transient nature, the demonstrated decrease in cytochrome P-450 would be expected to be sustained longer *in vivo*, which could contribute to the inhibition of the mixed function oxidase system by isoniazid. In support of these conclusions, preliminary studies showed that *in vivo* administration of an acute dose of isoniazid (100 mg/kg) injected i.p. 30 min before isolation of the rat liver microsomes resulted in a decrease in available cytochrome P-450.

Acknowledgement—Grateful acknowledgement is extended by the authors to Professor Samuel P. Bessman for his continuing support.

REFERENCES

1. P. Dineen, in *Drugs of Choice: 1971–1972* (Ed. W. Modell), p. 127. C. V. Mosby, St. Louis, MO (1972).
2. H. B. Hughes, *J. Pharmac. exp. Ther.* **109**, 444 (1953).
3. J. R. Mitchell, V. P. Thorgiesson, M. Black, J. A. Timbrell, W. R. Snodgrass, W. Z. Potter, D. J. Jollow and H. R. Keiser, *Clin. Pharmac. Ther.* **18**, 70 (1975).
4. J. H. Peters, K. S. Miller and P. Brown, *J. Pharmac. exp. Ther.* **150**, 298 (1965).
5. F. J. Murray, *Am. Rev. resp. Dis.* **86**, 729 (1962).
6. R. R. Miller, J. Porter and D. J. Greenblatt, *Chest* **75**, 3 (1979).
7. H. Kutt, R. Brennan, H. Dehejia and K. Verebely, *Am. Rev. resp. Dis.* **101**, 377 (1970).
8. H. S. Buttar, L. T. Wong and J. H. Moffatt, *Archs. int. Pharmacodyn. Thér.* **235**, 9 (1978).
9. G. Sutton and H. J. Kupferberg, *Neurology* **25**, 1179 (1975).
10. A. R. Rosenthal, T. H. Self, E. D. Baker and R. A. Linden, *J. Am. med. Ass.* **238**, 2177 (1977).
11. N. R. Eade, P. J. McLeod and S. M. MacLeod, *Am. Rev. resp. Dis.* **103**, 792 (1971).
12. S. F. Muakkassah, W. R. Bidlack and W. C. T. Yang, *Fedn. Proc.* **38**, 689 (1979).
13. H. Kutt, K. Verebely and F. McDowell, *Neurology* **18**, 706 (1968).
14. R. Kato, A. Takanaka and H. Shoji, *Jap. J. Pharmac.* **19**, 315 (1969).
15. J. A. Timbrell and J. M. Wright, *Drug Metab. Dispos.* **7**, 237 (1979).
16. D. A. Wolff and W. R. Bidlack, *Biochem. biophys. Res. Commun.* **73**, 850 (1976).
17. Y. Masuda and T. Murano, *Biochem. Pharmac.* **26**, 2275 (1977).
18. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
19. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
20. T. Nash, *Biochem. J.* **55**, 416 (1953).
21. Y. Imai, A. Ito and R. Sato, *J. Biochem., Tokyo* **60**, 417 (1966).
22. P. F. Thomas, A. Y. H. Lu, D. Ryan, S. B. West, J. Kawalek and W. Levin, *Molec. Pharmac.* **121**, 746 (1976).
23. W. Levin, D. Ryan, M. T. Huang, J. Kawalek, P. F. Thomas, S. B. West and A. Y. H. Lu, in *Microsomes and Drug Oxidation* (Eds. V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney), p. 185. Pergamon Press, Oxford (1977).
24. J. R. Fouts and B. B. Brodie, *J. Pharmac. exp. Ther.* **116**, 480 (1956).
25. W. W. Anders and G. J. Mannering, *Molec. Pharmac.* **2**, 319 (1966).
26. A. F. Welton and S. D. Aust, *Biochem. biophys. Res. Commun.* **49**, 661 (1972).
27. W. Levin, A. Y. H. Lu, M. Jacobson, R. Kuntzman, J. L. Poyer and P. B. McCay, *Archs Biochem. Biophys.* **158**, 842 (1973).
28. B. A. Schacter, H. S. Marver and U. A. Meyer, *Drug Metab. Dispos.* **1**, 286 (1973).
29. B. O. Christopherson, *Biochem. J.* **106**, 515 (1968).
30. E. D. Wills, *Biochem. J.* **113**, 315 (1969).
31. H. Kutt and K. Verebely, *Biochem. Pharmac.* **19**, 675 (1970).
32. I. Tadanobu, T. Umeda, L. Endrenyi, W. A. Mahon and W. Kalow, *Biochem. Pharmac.* **27**, 1151 (1978).
33. A. G. Hildebrandt, K. C. Liebman and R. W. Estabrook, *Biochem. biophys. Res. Commun.* **37**, 477 (1969).
34. D. V. Parke and H. Rahman, *Biochem. J.* **123**, 9p (1971).
35. C. R. Elcombe, J. W. Bridges, T. J. B. Gray, R. H. Nimmo-Smith and K. J. Netter, *Biochem. Pharmac.* **24**, 1427 (1975).
36. M. R. Franklin, *Xenobiotica* **1**, 581 (1971).
37. R. M. Philpot and E. Hodgson, *Life Sci.* **10**, 503 (1975).
38. R. M. Philpot and E. Hodgson, *Chem.–Biol. Interact.* **4**, 185 (1971/72).
39. M. R. Franklin, *Biochem. Pharmac.* **21**, 3287 (1972).
40. J. B. Schenkman, B. J. Wilson and D. L. Cinti, *Biochem. Pharmac.* **21**, 2373 (1972).
41. M. K. Buening and M. R. Franklin, *Drug Metab. Dispos.* **4**, 244 (1976).
42. J. Werringloer and R. W. Estabrook, *Life Sci.* **13**, 1319 (1973).
43. J. Werringloer and R. W. Estabrook, *Archs. Biochem. Biophys.* **167**, 270 (1975).
44. M. R. Franklin, *Xenobiotica* **4**, 133 (1974).
45. M. R. Franklin, *Molec. Pharmac.* **10**, 975 (1974).
46. M. R. Franklin, *Chem.–Biol. Interact.* **14**, 337 (1976).
47. M. R. Franklin, *Pharmac. Ther. (A)* **2**, 277 (1977).
48. R. A. Prough, *Archs Biochem. Biophys.* **158**, 442 (1973).
49. R. A. Prough, J. A. Wittkopp and D. J. Reed, *Archs. Biochem. Biophys.* **140**, 450 (1970).
50. R. A. Prough, M. L. Coomes and D. L. Dunn, in *Microsomes and Drug Oxidation* (Eds. V. Ullrich, I. Roots, A. Hildebrandt, R. W. Estabrook and A. H. Conney) p. 500. Pergamon Press, Oxford (1977).
51. J. Werringloer and R. W. Estabrook, in *The Induction of Drug Metabolism* (Eds. R. W. Estabrook and E. Lindenlaub), p. 269. Shattauer, Stuttgart (1979).