Biochemical Pharmacology, Vol. 36, No. 22, pp. 3873–3878, 1987.
Printed in Great Britain.

0006-2952/87 $3.00 + 0.00

EFFECTS OF POLY I:POLY C ON RAT PULMONARY AND HEPATIC CYTOCHROMES P-450 AND b_5*

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(*Received* 14 *October* 1986; *accepted* 6 *April* 1987)

Abstract—Interferon inducing agents such as poly I:poly C have been shown to reduce the hepatic hemoproteins cytochromes P-450 and b_5 along with the associated monooxygenase activities [el Azhary and Mannering, *Molec. Pharmac.* **15**, 698 (1979)]. In a previous study [Kikkawa *et al.*, *Lab. Invest.* **50**, 62 (1984)], we demonstrated that the interferon inducing agent poly I:poly C reduces pulmonary microsomal hemoprotein by 50% when administered to rats. The current investigation was conducted to characterize these changes in more detail and compare them to analogous changes in the liver. Compared to controls, cytochrome P-450 in both the lungs and livers of poly I:poly C treated rats declined by 40% at 24 hr and 55% at 48 hr ($P < 0.01$). By 72 hr the decline was only 25%. In contrast, cytochrome b_5 levels declined by less than 30% of control values during the first 48 hr following poly I:poly C injection ($P < 0.01$) and returned to control levels by 72 hr. These changes in both cytochrome P-450 and b_5 were reflected in decreases in pulmonary microsomal hemoprotein. Benzphetamine-*N*-demethylase activity declined by 45% in lung microsomes at 48 hr ($P < 0.01$) after injection of poly I:poly C, while 7-ethoxycoumarin-*O*-deethylase ($P < 0.05$) and 7-ethoxyresorufin-*O*-deethylase activities declined by approximately 41%. In the liver from these same poly I:poly C treated groups, benzphetamine-*N*-demethylase declined by 66% ($P < 0.05$), while 7-ethoxycoumarin-*O*-deethylase and 7-ethoxyresorufin-*O*-deethylase activities declined by 60% ($P < 0.02$ and $P < 0.05$ respectively).

The heme-containing cytochromes P-450 and b_5 are located in the endoplasmic reticulum. The former is associated with the monooxygenase system and xenobiotic metabolism, whereas the latter functions as an electron carrier from NADH cytochrome b_5 reductase to the fatty acid desaturase system and also to some cytochrome P-450 dependent monooxygenase reactions [1].

The levels of these cytochromes are depressed in microsomal preparations from the livers of rats treated with the interferon inducer poly I:poly C or Tilorone, in contrast to the levels of these cytochromes in other tissues, including the adrenal gland, kidney and intestinal mucosa, which remain unchanged [2, 3].

We previously demonstrated that injection of rats with the interferon inducer poly I:poly C leads to a decline in pulmonary microsomal hemoprotein of nearly 50% [4]; this decline coincides with an increase in heme oxygenase activity. Assuming that pulmonary microsomal hemoprotein represents primarily cytochromes P-450 and b_5, these results with the lung are comparable to those reported by el Azhary *et al.* [2, 3] for the liver. Since our previous study measured only changes in pulmonary microsomal hemoprotein, the present investigation was expanded to include measurements of cytochromes P-450 and b_5 along with the associated monooxygenase activities. These results were then compared to those obtained with the liver.

* This work was supported by USPHS Grants HL 34688 and HL 34109 from The National Institute of Health.

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MATERIALS AND METHODS

Treatment of animals. Male, Sprague–Dawley CD strain, specific pathogen free, virus free rats (250–300 g) were purchased from Charles River, Willmington, MA, and were housed in rooms containing positive pressure, filtered, laminar flow ventilation.

Poly I:poly C, sodium salt, in a 0.9% saline solution at a concentration of 10 mg/kg was injected i.p. into rats [3, 4]. Controls received saline alone.

Microsomal isolation. Rats were anesthesized with an i.p. injection of pentobarbital (30 mg/kg), and body weight was recorded. The lungs and livers were then simultaneously perfused with 0.9% saline via the hepatic portal vein [4]. This approach enabled the chest cavity to remain closed, making complete perfusion of the lungs more feasible. For each animal, approximately 200 ml of saline was used.

When perfusion was completed, the lungs and liver were removed and weighed. Tissues were then homogenized in 1.15% KCl, 0.05 M Tris–HCl, pH 7.4 (7 ml/g wet weight) in a microblender for 30 sec followed by 15 strokes with a Potter–Elvehjem Teflon–glass homogenizer. The volumes of the homogenate fractions were then recorded, and samples were taken for use in enzyme assays. The remainders of the fractions were spun at 1,000 *g* for 5 min, and the resulting supernatant fractions were then spun successively at 10,000 *g* for 15 min and at 105,000 *g* for 60 min. The microsomal pellets were resuspended in homogenization medium and centrifuged again at 105,000 *g* for 60 min and then resuspended in homogenization buffer. Samples were then taken for protein determination and for various biochemical analyses described below.

Biochemical analyses. Cytochromes P-450 and b_5 were determined according to the method of Omura and Sato [5] using an Aminco DW-2C spectrophotometer. Lung and liver microsomes were first diluted to concentrations of 1.5 to 2 mg/ml using 0.1 M potassium phosphate buffer, pH 7.4.

Glucose-6-phosphatase and 5′-nucleotidase assays were performed on both homogenates and microsomal preparations as described by Aronson and Touster [6]; acid phosphatase was done as described by Trouet [7]. In these assays inorganic phosphate was used as a standard. All assays were performed with a Beckman model 35 spectrophotometer.

Succinate cytochrome *c* and NADPH–cytochrome *c* reductase activities were determined in both homogenates and microsomal preparations using the methods of Sottocasa *et al.* [8] and Strobel and Dignam [9] respectively. Assays were done with a Gilford 2400 spectrophotometer. Protein determination was done as described previously [4].

Benzphetamine-*N*-demethylase activity was determined in both rat lung and liver microsomes by determining the amount of formaldehyde generated as described by Werringloer [10]. The O-dealkylations of 7-ethoxycoumarin and 7-ethoxyresorufin were done on both lung and liver microsomes by measuring the fluorescence of the dealkylated product, 7-hydroxycoumarin or 7-hydroxyresorufin. These assays were done as described by Prough *et al.* [11] using a fluorescence attachment to the Aminco DW2C spectrophotometer.

Microsomal heme content was measured on lung and liver microsomes spectrophotometrically after conversion of heme into the pyridine hemochromogen in the presence of 0.1 N NaOH and 20% pyridine [5].

Data expression and statistics. Enzyme activities for glucose-6-phosphatase, 5′-nucleotidase, acid phosphatase, succinate cytochrome *c* and NADPH–cytochrome *c* reductases are expressed as a percentage of microsomal activity over total homogenate activity, or on a per gram of lung or liver basis. Cytochromes P-450 and b_5 and heme content are on a per mg microsomal protein basis. Data are expressed as mean ± 1 SD of at least three determinations, and a 0.05 confidence level, determined by Student's *t*-test, is taken to indicate statistical significance.

Reagents. Poly I:poly C, benzphetamine, 7-ethoxycoumarin, 7-hydroxycoumarin (umbelliferone) and 7-hydroxyresorufin were obtained through the Sigma Chemical Co., St. Louis, MO. 7-Ethoxyresorufin was purchased through Molecular Probes, Junction City, OR.

RESULTS

Assays as a function of time are shown in Table 1A for the lung and 1B for the liver. Enzyme levels

Table 1A. Yield and total activity of various marker enzymes in microsomal fractions and whole lung from control and poly I:poly C treated rats

Enzyme	Enzyme organelle marker		Hours after injection 24	48	72
				Microsomal yield*	
Glucose-6-phosphatase	Microsomes	Control	14.6 ± 1.7	12.2 ± 1.4	15.0 ± 2.2
		Poly I:Poly C	14.4 ± 1.4	14.6 ± 2.7	13.8 ± 3.6
Succinate cytochrome *c* reductase	Mitochondria	Control	1.0 ± 0.1	1.9 ± 0.1	2.2 ± 0.3
		Poly I:Poly C	0.9 ± 0.2	1.6 ± 0.4	2.2 ± 0.4
Acid phosphatase	Lysozomes	Control	12.7 ± 1.3	9.5 ± 0.5	15.7 ± 1.6
		Poly I:Poly C	12.9 ± 1.0	9.9 ± 1.4	17.2 ± 6.6
5′-Nucleotidase	Plasma membrane	Control	8.5 ± 1.6	11.0 ± 0.4	6.9 ± 11.3
		Poly I:Poly C	7.0 ± 0.5	9.9 ± 0.9	7.6 ± 1.9
NADPH–cytochrome *c* reductase	Microsomes	Control	22.8 ± 5.3	31.0 ± 1.9	19.5 ± 2.5
		Poly I:Poly C	25.3 ± 4.6	25.9 ± 2.6	20.4 ± 2.8
				Total activity†	
Glucose-6-phosphatase	Microsomes	Control	1.7 ± 0.1	1.4 ± 0.2	2.0 ± 0.1
		Poly I:Poly C	1.9 ± 0.1	1.4 ± 0.2	2.4 ± 0.5
Succinate cytochrome *c* reductase	Mitochondria	Control	476.7 ± 93.6	570.8 ± 109.7	633.3 ± 62.2
		Poly I:Poly C	421.5 ± 33.2	606.4 ± 35.5	743.4 ± 23.0
Acid phosphatase	Lysozomes	Control	2.1 ± 0.1	2.4 ± 0.2	2.6 ± 0.6
		Poly I:Poly C	2.3 ± 0.2	2.4 ± 0.1	3.0 ± 0.6
5′-Nucleotidase	Plasma membrane	Control	3.3 ± 0.1	4.8 ± 0.5	3.6 ± 0.6
		Poly I:Poly C	3.0 ± 0.5	3.7 ± 0.2	3.2 ± 0.6
NADPH–cytochrome *c* reductase	Microsomes	Control	1567.0 ± 87.0	1342.3 ± 123.4	1080.8 ± 94.0
		Poly I:Poly C	1394.2 ± 126.0	1572.2 ± 378.1	1001.8 ± 203.5

* Values represent the percentage of total homogenate activity and are mean ± SD, N = 3.

† Values represent enzyme activity on a per gram lung basis. Glucose-6-phosphatase, acid phosphatase and 5′-nucleotidase activities are expressed as μmol PO_4 released/min/g; NADPH and succinate cytochrome *c* reductase are expressed as nmol cytochrome *c* reduced/min/g. Values are mean ± SD, N = 3.

Table 1B. Yield and total activity of marker enzymes in both hepatic microsomes and liver from control and poly I:poly C treated rats

Enzyme	Enzyme organelle marker		Hours after injection 24	48	72
				Microsomal yield*	
Glucose-6-phosphatase	Microsomes	Control	19.2 ± 2.6	28.1 ± 2.8	24.4 ± 4.0
		Poly I:Poly C	19.1 ± 2.5	21.1 ± 4.3	21.5 ± 2.6
Succinate cytochrome *c* reductase	Mitochondria	Control	0.3 ± 0.1	0.3 ± 0.1	0.9 ± 0.2
		Poly I:Poly C	0.3 ± 0.1	0.3 ± 0.1	0.4 ± 0.2
Acid phosphatase	Lysozomes	Control	18.9 ± 2.2	20.0 ± 1.3	23.9 ± 6.6
		Poly I:Poly C	17.7 ± 0.5	11.5 ± 4.7	18.0 ± 5.6
5′-Nucleotidase	Plasma membrane	Control	6.0 ± 1.4	7.7 ± 1.2	7.3 ± 2.1
		Poly I:Poly C	5.4 ± 2.1	9.6 ± 1.5	6.9 ± 2.2
NADPH–cytochrome *c* reductase	Microsomes	Control	30.4 ± 3.2	29.2 ± 6.0	26.6 ± 4.3
		Poly I:Poly C	27.1 ± 1.4	31.8 ± 9.4	33.6 ± 4.8
				Total activity†	
Glucose-6-phosphatase	Microsomes	Control	14.5 ± 0.9	16.4 ± 1.9	19.4 ± 4.9
		Poly I:Poly C	19.7 ± 3.7	18.8 ± 3.0	21.8 ± 1.8
Succinate cytochrome *c* reductase	Mitochondria	Control	2912.2 ± 432.4	3871.5 ± 432.2	2994.3 ± 390.4
		Poly I:Poly C	2260.1 ± 476.6	3240.6 ± 357.7	3419.3 ± 950.5
Acid phosphatase	Lysozomes	Control	6.0 ± 0.6	5.6 ± 0.8	4.8 ± 0.4
		Poly I:Poly C	5.5 ± 0.2	5.3 ± 1.8	5.3 ± 0.6
5′-Nucleotidase	Plasma membrane	Control	9.6 ± 2.7	11.8 ± 1.2	9.5 ± 2.2
		Poly I:Poly C	9.8 ± 1.5	13.2 ± 2.8	10.0 ± 2.4
NADPH–cytochrome *c* reductase	Microsomes	Control	3246.1 ± 264.4	2641.6 ± 409.6	2965.5 ± 21.7
		Poly I:Poly C	2940.2 ± 222.6	2041.3 ± 459.5	2234.9 ± 57.9

* Values represent the percentage of total homogenate activity and are mean ± SD, N = 3.

† Values represent enzyme activity on a per gram lung basis. Glucose-6-phosphatase, acid phosphatase and 5′-nucleotidase are expressed as μmol PO_4 released/min/g liver; NADPH and succinate cytochrome *c* reductase are expressed as nmol cytochrome *c* reduced/min/g. Values are mean ± SD, N = 3.

are expressed both as a percentage of total microsomal yield and as total activities of the tissue homogenate. The results show that, for a specific enzyme, the percentage yields in the microsomes and total activities were similar at a given time point for both control and poly I:poly C treated groups. For example, in the lung, at 24 hr, the percent activity of the plasma membrane marker, 5′-nucleotidase, was approximately 7% in both groups, while total activity in each was approximately 3.0 μmol PO_4 released/min/g lung. From these results, it is reasonable to assume that microsomal preparations from control and experimental groups were similar in terms of contamination.

Table 2 presents the yields recovered from microsomes in milligrams per gram of lung or per gram of liver from the experimental and control groups. For the lung, these yields were approximately 5 mg/g, whereas for the liver they were approximately 20 mg/g.

The data from Tables 1A, 1B and 2 indicate that, at a given time point, the microsomes isolated from either the lungs or livers of control and poly I:poly C treated rats were similar in terms of enzyme marker profiles and in the amounts of microsomal protein recovered. In subsequent experiments, this similarity allowed comparison of changes in components of the monooxygenase system in controls and poly I:poly C treated rats to be made on a per milligram microsomal protein basis.

The effects on the levels of the cytochromes P-450 and b_5 and the total heme protein in microsomes

Table 2. Yield of recovered microsomal protein to lung and liver

		Microsomal protein (mg/g lung or liver) Hours after injection 24	38	72
Lung	Control	5.5 ± 0.6	4.5 ± 0.4	4.8 ± 0.2
	Poly I:Poly C	4.5 ± 0.9	4.6 ± 0.5	5.5 ± 0.4
Liver	Control	23.2 ± 4.7	15.5 ± 2.0	20.3 ± 1.5
	Poly I:Poly C	19.9 ± 3.7	20.0 ± 1.1	19.3 ± 1.2

Values are mean ± SD, N = 3.

Table 3. Content of cytochromes P-450 and b_5 and heme from microsomes isolated from lungs and livers of control and poly I:poly C treated rats

		Content (nmol/mg microsomal protein)		
		Hours after injection		
		24	48	72
		Cytochrome P-450		
Lung	Control	0.045 ± 0.010	0.052 ± 0.007	0.049 ± 0.009
	Poly I:Poly C	0.027 ± 0.006	0.024 ± 0.005*	0.035 ± 0.008
Liver	Control	0.780 ± 0.100	0.755 ± 0.100	0.722 ± 0.113
	Poly I:Poly C	0.455 ± 0.082†	0.340 ± 0.070*	0.548 ± 0.009
		Cytochrome b_5		
Lung	Control	0.043 ± 0.007	0.048 ± 0.006	0.044 ± 0.002
	Poly I:Poly C	0.030 ± 0.005	0.031 ± 0.004*	0.041 ± 0.001
Liver	Control	0.386 ± 0.078	0.376 ± 0.080	0.355 ± 0.064
	Poly I:Poly C	0.275 ± 0.060	0.260 ± 0.090	0.360 ± 0.018
		Heme		
Lung	Control	0.117 ± 0.019	0.111 ± 0.015	0.120 ± 0.030
	Poly I:Poly C	0.075 ± 0.023	0.071 ± 0.020†	0.092 ± 0.023
Liver	Control	1.370 ± 0.198	1.4 ± 0.100	1.21 ± 0.168
	Poly I:Poly C	0.900 ± 0.058†	0.74 ± 0.040*	0.95 ± 0.115

Values are mean ± SD; N = 4 for lung and N = 3 for liver.
* $P < 0.01$, compared to control.
† $P < 0.05$, compared to control.

isolated from the lungs and livers of rats following injection of poly I:poly C are shown in Table 3. The content of cytochrome P-450 in the lungs and livers declined by 40% during the first 24 hr following poly I:poly C injection; during the next 24 hr it declined to approximately 55%, which was significant in both the lung and liver ($P < 0.01$); by 72 hr, however, the levels had recovered to 71 and 75% of the control values in the lung and liver respectively. The decline of cytochrome b_5 was statistically significant in the lung and liver at 48 hr ($P < 0.01$) with a decrease of 35%, compared to 48-hr controls. Total microsomal heme protein also declined significantly ($P < 0.05$) for both the lung and liver at 48 hr, reflecting the changes seen with cytochromes P-450 and b_5.

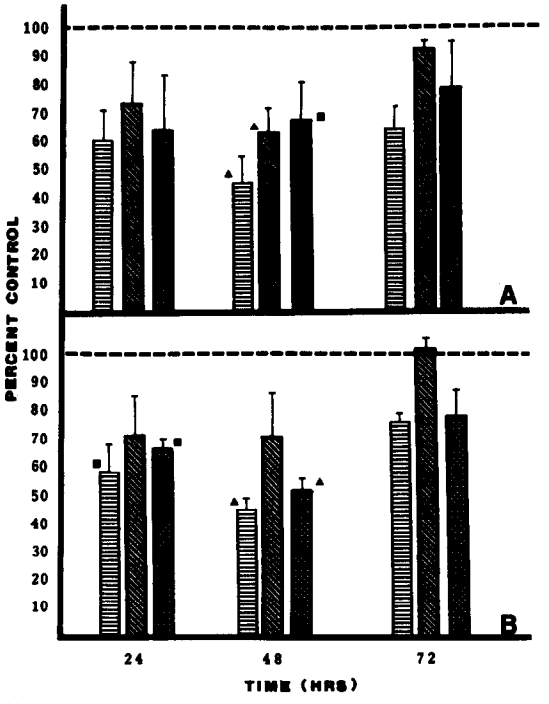

Fig. 1. Percent change compared to controls of cytochromes P-450 and b_5 and total microsomal heme protein from the lungs (A) and livers (B) of poly I:poly C treated rats. Key: ▤ cytochrome P-450, ▧ cytochrome b_5, ▨ heme; (■) $P < 0.05$, and (▲) $P < 0.01$, compared to 100% control values (see Table 3 for control values). Values are mean ± SD, N = 4 for lung and N = 3 for liver.

The percent changes in cytochromes P-450 and b_5 and total heme content in the lung and liver microsomes from the poly I:poly C treated rats as compared to the controls are shown in Fig. 1. The similarities in the declines of these components between the lung and the liver were evident both in terms of percent decline and in their time course.

Of the three time points studied, maximal depression of cytochrome P-450 occurred at 48 hr. Therefore, this time was chosen to examine changes in specific P-450 associated monooxygenase activities from microsomes obtained from the lungs and livers of poly I:poly C treated rats (Table 4). Compared to controls, benzphetamine-*N*-demethylase activity declined significantly by approximately 50% in both lung ($P < 0.01$) and liver ($P < 0.05$) microsomes from poly I:poly C treated rats. 7-Ethoxycoumarin *O*-deethylase activity declined significantly by 40% in lung ($P < 0.05$) and 60% in liver ($P < 0.02$) microsomal preparations. The same trend was also seen in the decline of 7-ethoxyresorufin-*O*-deethylase activity. In the microsomal preparations from the lungs of poly I:poly C treated animals, this decline was 43% but was not significant, whereas for the liver this was 52% and was significant ($P < 0.01$).

Table 4. Effects of poly I:poly C at 48 hr on benzphetamine-*N*-demethylase, 7-ethoxycoumarin-*O*-deethylase, and 7-ethoxyresorufin-*O*-deethylase activities in microsomes from rat lung and liver

		Benzphetamine-*N*-demethylase (nmol/HCHO formed/min/mg protein)	7-Ethoxycoumarin-*O*-deethylase (nmol umbelliferone/min/mg protein)	7-Ethoxyresorufin *O*-deethylase (pmol 7-hydroxyresorufin/min/mg protein)
Lung	Control	0.19 ± 0.02	1.30 ± 0.28	2.50 ± 0.56
	Poly I:Poly C	0.11 ± 0.01*	0.77 ± 0.07†	1.43 ± 0.64
Liver	Control	0.28 ± 0.05	4.00 ± 0.86	23.36 ± 2.25
	Poly I:Poly C	0.14 ± 0.002†	1.61 ± 0.15‡	11.30 ± 4.50†

Values are mean ± SD; N = 4 for lung and N = 3 for liver.
* $P < 0.01$, compared to control.
† $P < 0.05$, compared to control.
‡ $P < 0.02$, compared to control.

DISCUSSION

In this report, it was demonstrated that cytochromes P-450 and b_5 were depressed in the rat lung following the administration of the interferon inducer, poly I:poly C. The decline of cytochromes P-450 and b_5 in the lung paralleled similar declines in cytochromes P-450 and b_5 in the rat liver, which agrees with the earlier findings reported by el Azhary *et al.* [2, 3]. In a previous investigation [4], we showed that the administration of poly I:poly C to rats results in a decline of microsomal hemoprotein in the rat lung. In the present study, cytochromes P-450 and b_5 together constituted approximately 75–80% of the microsomal hemoprotein in the rat lung and 85–90% in the rat liver. These ratios held true for both control and poly I:poly C treated rats. The remaining hemoprotein was not characterized but could consist of other microsomal hemoproteins such as cyclooxygenase and heme oxygenase. Other contaminating non-microsomal hemoproteins including mitochondrial cytochromes and hemoglobin were probably minimal. Mitochondrial contamination as judged by the activity of succinate cytochrome *c* reductase was quite low, less than 2% in the lung and less than 1% in the liver.

Our studies comparing changes in the levels of various organelle marker enzymes in both the lung and liver agree with those reported for the mouse liver by Gooderham and Mannering [12]. These investigators reported that glucose-6-phosphatase, 5′-nucleotidase and acid phosphatase levels were not affected by treatment of mice with poly I:poly C. In contrast to our results, however, they report a slight increase in the level of succinate cytochrome *c* reductase.

The procedure used for preparation of the microsomes was designed to minimize the amount of hemoglobin contamination. It included perfusion of the lungs via the hepatic portal vein with the chest closed until they appeared white. This was followed by use of a 1.15% KCl, 0.05 M Tris–HCl homogenization and washing medium. With this approach there was no detectable hemoglobin found in carbon monoxide difference spectra [13], nor was methemoglobin found to interfere in determining cytochrome P-450 levels [13]. Thus, our previous notion that the depression of hemoprotein represented a decline in cytochrome P-450 can be justified from this study.

Cytochrome P-450 in the lung is composed primarily of two isozymes. Using rabbit lung nomenclature [14], these are forms 2 and 5. In the rat lung, the same two forms have been shown to exist although the nomenclature is different [14]. Form 2 is similar to the phenobarbital inducible form of the liver but, unlike the liver, is not induced by phenobarbital [15]. Several investigations have shown that benzphetamine-*N*-demethylase and 7-ethoxycoumarin-*O*-deethylase monooxygenase activities are associated only with form 2 in the lung [16]. The second major form found in the lung, form 5, has not been characterized as extensively. Its monooxygenase activities are directed towards formation of mutagenic products as determined by the salmonellae assay devised by Ames *et al.* [17]. Like

form 2, it is also not inducible. A third isozyme of cytochrome P-450, form 6, is normally found only in small quantities in the lung [18]. Unlike the other forms found in the lung, it is inducible by treatment of an animal with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) or benzo[*a*]pyrene. It has been established that the metabolism of 7-ethoxyresorufin to the 7-hydroxy form is specific for form 6 [16]. Thus, measurement of the activities of benzphetamine-*N*-demethylase, 7-ethoxycoumarin-*O*-deethylase and 7-ethoxyresorufin-*O*-deethylase can provide information on specific changes in the activities of cytochrome P-450 isozyme levels, with treatment by poly I:poly C.

In the results shown in Table 3, the depression of cytochrome P-450 at 48 hr was 54% for the lung and 55% for the liver. The three monooxygenase activities appeared to decline uniformly for the lung, being approximately 42% (Table 4). In the liver this decline appeared to be slightly greater than the level found in the lung. Thus, we were not able to detect any preferential depression of pulmonary cytochrome P-450 isozymes with respect to forms 2 and 6. Other investigators [19, 20] have shown that isozymes of cytochrome P-450 of the liver are depressed in different proportions.

el Azhary *et al.* [2, 3] reported that treatment of rats with the interferon inducer poly I:poly C leads to an induction of heme oxygenase prior to the decline of cytochromes P-450 and b_5. In a previous study [4], we found that poly I:poly C treatment also causes an induction of heme oxygenase in the lung, prior to a decline in pulmonary microsomal hemoprotein. These results suggest that the lung reacts to interferon inducers in a manner similar to that described for the liver by el Azhary *et al.* [2, 3].

Recently, the lung has become a focus for the study of environmental toxins and carcinogens. The ability of interferon inducers to reduce cytochrome P-450 and its associated monooxygenase activities has several interesting implications. First, this could be used as a system to contrast with those substances known to induce cytochrome P-450 in the lung. Second, if the depression of cytochrome P-450 in the lung is a common property of interferon inducers, the implication in the study of human lung and its reaction to toxins and xenobiotics can be profound.

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