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CATALYTIC AND IMMUNOLOGIC CHARACTERIZATION OF HEPATIC AND LUNG CYTOCHROMES P450 IN THE POLAR BEAR

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Abstract—The Arctic Ocean is subject to considerable influx of anthropogenic pollutants including halogenated organic compounds. The polar bear (*Ursus maritimus*) is at the top of the arctic marine food web and is an ideal species for monitoring the level and distribution of contaminants in the arctic ecosystem. As the first step in the development of a biological method for assessing the functional exposure of polar bears to xenobiotics, biochemical studies were undertaken to characterize polar bear cytochromes P450. Liver and lung samples were obtained in the field from four, freshly killed, adult, male polar bears and immediately frozen at −196°. Microsomes were subsequently prepared and used for the measurement of total cytochrome P450 content and aminopyrine *N*-demethylase, benzphetamine *N*-demethylase, ethylmorphine *N*-demethylase, *p*-nitrophenol hydroxylase and testosterone hydroxylase activities. Immunoblots containing hepatic and lung microsomal samples from the polar bears were probed using antibodies generated against several purified rat cytochrome P450 isozymes. Monoclonal antibody to rat cytochrome P450 1A1 and polyclonal antibodies to rat cytochromes P450 1A1, 2B1 and 3A1, as well as antibody to epoxide hydrolase, cross-reacted to varying degrees with polar bear hepatic microsomes. In addition, polyspecific antibody to the rat cytochrome P450 2C subfamily gave several immunostained protein bands, but antibodies specific to rat cytochrome P450 2C7 and 2C13 did not react, while antibody specific to cytochrome P450 2C11 yielded an ambiguous result. Except for anti-cytochrome P450 2B1 and polyspecific antibody to the cytochrome P450 2C subfamily, the antibodies listed above did not cross-react with polar bear lung microsomes at the protein concentrations used. The results demonstrate that polar bear liver contains multiple forms of cytochrome P450 that are catalytically active toward diverse substrates and that several of these forms are immunochemically related to rat cytochrome P450 isozymes. Immunochemical homologues of rat cytochrome P450 1A, 2B, 2C and 3A subfamilies, and of rat epoxide hydrolase are present in polar bear liver. In addition, the polar bears all had high levels of immunoreactive cytochrome P450 1A and 2B proteins, probably as a consequence of induction by environmental contaminants.

Key words: cytochrome P450; polar bear; hepatic; microsomal monooxygenases; immunochemical relatedness; arctic ecosystem

Cytochromes P450 are a large and ubiquitous group of enzymes found in mammals, birds, fish and microorganisms [1–8]. These enzymes function in the conversion of lipophilic foreign compounds, including most therapeutic drugs, pesticides and environmental pollutants, into more water-soluble metabolites for subsequent excretion. Considerable information on cytochrome P450 enzymes is available for laboratory animals such as the rat, mouse and rabbit, but much less is known about this important family of enzymes in wild species such as the polar bear. From a toxicological perspective, characterization of hepatic cytochromes P450 in free-ranging animals is important for several reasons. First, the multiplicity of these enzymes and their diverse substrate specificities endow organisms with the ability to metabolize and detoxify xenobiotic compounds. Moreover, cytochromes P450 occur in the highest concentration in liver, which is the primary site for the bioaccumulation and biotransformation of lipophilic chemicals. Second, several cytochrome P450 isozymes are induced by a variety of foreign chemicals. Because hepatic cytochromes P450 play a critical role in metabolism and, therefore, in the bioaccumulation and potential toxicity of many environmental contaminants found in the food chain, the level and inducibility of individual isozymes are important determinants of susceptibility to environmental contaminant exposure. Third, important qualitative and quantitative differences in the presence, activity and inducibility of cytochromes P450 have been reported for different species [9–12]. Lastly, cytochrome P450

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‖ Abbreviations: HCHO, formaldehyde; HRP, horseradish peroxidase; IgG, immunoglobulin G; MC, 3-methylcholanthrene; PB, phenobarbital; PCBs, polychlorinated biphenyls; and Ut, untreated.

levels in exposed animals may be useful as potential indicators of environmental contamination.

The polar bear (*Ursus maritimus*) is distributed widely throughout the arctic circumpolar regions and can be a valuable sentinel species for monitoring arctic marine ecosystem health. Its range approximates, to a very high degree, the maximum extent of sea ice in winter, and individual bears move over vast regions of the Arctic Ocean in search of food [13, 14]. Polar bears feed primarily on ringed seals (*Phoca hispida*), which in turn feed on arctic cod (*Boreogadus saida*) and large amphipods [15]. Thus, polar bears are at the top of the arctic marine food chain. Because the polar bear diet is very high in fat and because fat is the depot for the accumulation of lipophilic xenobiotics, environmental contaminants such as PCBs‖ and other halogenated organic compounds [16–18] are biomagnified in these animals. For example, mean PCB concentrations reported for pooled adipose tissue of polar bears from the Canadian arctic were from six to fifteen times greater than those measured in the blubber of ringed seals from the same geographic location [19]. Furthermore, PCB levels greater than about 70 μg/g of fat have been reported for some polar bears from both Canada and Norway [16, 18], and tissue PCB concentrations in excess of this threshold value have been linked to reproductive failures in other marine mammals [20–24]: In addition to having high levels of PCBs and other chlorinated contaminants, polar bears undergo seasonal periods of fasting that can last for months, during which time their body mass can decrease by more than half with concurrent reduction in adipose tissue reserves from approximately 40–50% of body mass to less than 10% [25, 26]. During the fasting period, PCB concentrations in liver can be expected to increase in proportion to loss of fat [27]. Consequently, polar bears may be at increased potential risk from PCBs and related compounds.

To determine if measurement of cytochrome P450 levels in free ranging polar bears is useful as a potential indicator of environmental contamination, fundamental information about the cytochrome P450 composition of polar bear tissue, as well as the inducibility and properties of these enzymes, is needed. Polar bear liver samples are routinely available through aboriginal hunters, but it is logistically difficult to obtain liver samples in a manner that preserves catalytic activity. Therefore, a long-term goal of our research is to investigate the possibility of developing techniques for immunoquantitation of cytochromes P450 in frozen liver tissue as a measure of exposure to halogenated organic compounds. The purpose of the present preliminary study was to identify and characterize cytochromes P450 in polar bear liver and lung microsomal preparations using enzymatic activities and antibodies prepared against individual purified rat cytochrome P450 isozymes. Antibodies, in particular, have proven to be very useful for evaluating the structural relatedness of different cytochrome P450 isozymes in both intraspecies and interspecies studies [28–31], in that proteins with similar amino acid sequences cross-react in immunological tests with multivalent antibody prepared against one of the proteins, and this cross-reactivity does not disappear until there is approximately a 40% difference in amino acid sequence [32, 33].

MATERIALS AND METHODS

Chemicals. BSA (98% fatty acid free) and diaminobenzidine were purchased from ICN Biochemicals (St. Laurent, Quebec). Acrylamide, sodium carbonate, acetic acid, potassium phosphate, magnesium chloride, zinc sulfate, sodium hydroxide, semicarbazide hydrochloride, phenol (Folin and Ciocalteu) reagent, nickel chloride, glycine and ammonium persulfate were obtained from BDH Chemicals (Toronto, Ontario). *N,N,N′,N′*-Tetramethylenediamine (TEMED) was bought from Bio-Rad Laboratories, Canada (Mississauga, Ontario). HPLC-grade dichloromethane, HPLC-grade methanol, barium hydroxide, acetylacetone, ammonium acetate, ascorbic acid, perchloric acid, formaldehyde [37% (w/w) solution] tris(hydroxymethyl)-aminoethane (Tris base) and 4-chloro-1-naphthol were purchased from Fisher Scientific (Vancouver, British Columbia). NADPH, tetrasodium salt, was obtained from Boehringer Mannheim Canada (Laval, Quebec). Cupric sulfate, dimethylaminoantipyrine, *p*-nitrophenol and 1,2-dihydroxy-4-nitrobenzene were bought from the Sigma Chemical Co. (St. Louis, MO). Sodium dithionite was purchased from J. T. Baker Inc. (Phillipsburg, NJ). Nitrocellulose membrane was obtained from Schleicher & Schuell (Keene, NH). Affinity-isolated goat F(ab′)2 anti-rabbit immunoglobulin (gamma and light chain specific, human IgG adsorbed) HRP-conjugated antibodies and affinity-isolated goat anti-mouse immunoglobulin (gamma and light chain specific) HRP-conjugated antibodies were purchased from TAGO Immunologicals Inc. (Inter-Medico, Markham, Ontario). Hydrogen peroxide [30% (w/v) solution] was obtained from Anachemia (Montreal, Quebec). Testosterone, androstenedione and 2α-, 2β-, 6β-, 7α-, 11β-, 16α- and 16β-hydroxytestosterones were bought from Steraloids Inc. (Wilton, NH). Benzphetamine hydrochloride and ethylmorphine hydrochloride dihydrate were provided by the Bureau of Dangerous Drugs, Health and Welfare Canada (Ottawa, Ontario).

Animal treatment and preparation of microsomes. Tissue samples from four adult male polar bears, ranging in age from 5 to 22 years, were collected in a controlled hunt in April 1992 in the Canadian arctic near Resolute, Northwest Territories. The collection of tissue samples took place as part of a separate project designed to study the half-life of the immobilizing drug, Telazol®, in polar bears. Telazol® is a 1 : 1 mixture of tiletamine hydrochloride and zolazepam hydrochloride (A. H. Robins Co., Richmond, VA). The bears were immobilized by a Telazol® dart and radio-tagged. One to five days later, the bears were located by helicopter and shot by Inuit hunters as part of their annual quota. Liver and lung samples were removed by biologists within 10–15 min after death, cut into small pieces, placed in labeled vials and frozen at −196° in liquid nitrogen. Five months later, the frozen liver and lung samples

were thawed, and hepatic and lung microsomal fractions were prepared by differential centrifugation as described by Thomas *et al.* [34]. The microsomal pellets were suspended in 0.25 M sucrose, and aliquots of the suspension were stored at −80° until used.

Hepatic microsomes were also prepared from adult male Long Evans rats that were either untreated or treated with sodium phenobarbital (75 mg/kg body weight/day, i.p., for 4 days) or MC (25 mg/kg body weight/day, i.p., for 4 days). For each treatment group, hepatic microsomes were prepared and pooled from at least five rats.

Determination of cytochrome P450 and protein. Total cytochrome P450 content was determined from the carbon monoxide reduced difference spectrum using the method of Omura and Sato [35]. Protein concentration was determined by the method of Lowry *et al.* [36].

Enzyme assays. The N-demethylations of aminopyrine, benzphetamine and ethylmorphine were measured separately using the same spectrophotometric assay. HCHO, a product of the demethylation reactions, was trapped in the reaction mixture by semicarbazide and subsequently estimated colorimetrically by means of the Hantzsch reaction as described by Nash [37]. Reaction mixtures contained 0.1 M potassium phosphate buffer, pH 7.5, 10 mM magnesium chloride, 5 mM semicarbazide hydrochloride, 5 mM substrate (either dimethylaminoantipyrine, benzphetamine hydrochloride or ethylmorphine hydrochloride dihydrate), 0.5 mM NADPH and 1.0 mg of microsomal protein in a final volume of 2.0 mL. The reactions were initiated with NADPH after preincubation for 5 min at 37° and were terminated, after a 10-min incubation at 37°, by the addition of 1.0 mL of 5% (w/v) zinc sulfate and 1.0 mL of saturated barium hydroxide. Tubes were mixed immediately and then spun in a centrifuge at 2000 × *g* for 15 min. The amount of HCHO formed was determined in 2.0 mL of the clear supernatant after the addition of 2.0 mL of double-strength Nash reagent, followed by a 60-min incubation at 37° to develop the yellow color. The tubes were allowed to cool to room temperature before the absorbance at 412 nm was measured. The amount of HCHO present was calculated from a previously prepared standard curve.

The hydroxylation of *p*-nitrophenol to 4-nitrocatechol (1,2-dihydroxy-4-nitrobenzene) was determined spectrophotometrically as described by Koop [38]. Reaction mixtures contained 0.1 M potassium phosphate buffer, pH 6.8, 5 mM magnesium chloride, 0.1 mM *p*-nitrophenol, 1.0 mM ascorbic acid, 0.5 mM NADPH and 4.0 mg of microsomal protein in a final volume of 2.0 mL. The reactions were initiated with NADPH after preincubation for 10 min at room temperature and were terminated with 1.0 mL of 6 M perchloric acid after a 5-min incubation at 37°. Tubes were mixed immediately and then spun in a centrifuge at 2000 × *g* for 10 min. The amount of 4-nitrocatechol formed was determined spectrally in 2.5 mL of the supernatant after the addition of 0.2 mL of 10 M NaOH. The tubes were allowed to sit for 15 min at room temperature, and then the absorbance at 546 nm was measured. The amount of 4-nitrocatechol present was calculated from a previously prepared standard curve.

Additional experiments were conducted with polar bear hepatic microsomes to ensure that the rate of product formation was linear with respect to both incubation time and microsomal protein concentration for the hydroxylation of *p*-nitrophenol, as well as for the N-demethylations of aminopyrine, benzphetamine and ethylmorphine.

The microsomal oxidation of testosterone was measured using the method of Sonderfan *et al.* [39], with a few modifications. Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 3 mM magnesium chloride, 1.0 mM NADPH, 0.25 mM testosterone and 0.3 mg of microsomal protein in a final volume of 1.0 mL. Reactions were initiated with testosterone after preincubation for 10 min at room temperature and were terminated by the addition of 6.0 mL dichloromethane after a 5-min incubation at 37°. A fixed amount of internal standard, 2.5 nmol 11β-hydroxytestosterone, was added to each tube. Tubes were mixed vigorously for several minutes and then spun in a centrifuge at 2000 *g* for 5 min. The aqueous phase was aspirated and discarded, and all of the remaining organic phase was evaporated to dryness under a gentle stream of nitrogen. The residue was dissolved in 0.20 mL of methanol with vigorous mixing and then filtered through a 3-mm, 0.45 μm, syringe filter. A 10-μL aliquot was analyzed by HPLC. The HPLC system, including the instrument, columns, solvent and elution conditions, was the same as that described by Sonderfan *et al.* [39]. Metabolites were identified by comparing retention times with those of authentic standards. The amount of each metabolite formed was calculated from the slope of a linear calibration curve using the ratio of the peak area (integrated by a Shimadzu CR501 chromatography data processor) of the metabolite to that of the internal standard. Calibration curves were generated for each metabolite by plotting the ratio of the peak area of the authentic standard to that of the internal standard versus the concentration of the authentic standard. Calibration curves were prepared as part of every assay and employed four concentrations of each authentic standard. Tubes to which authentic standards were added contained the complete reaction mixture except for microsomal protein.

Preparation of antibodies. Polyclonal antibodies against rat cytochrome P450 2B1, 2C7, 2C11, 2C13 and 3A1, as well as rat epoxide hydrolase, were raised separately in female New Zealand rabbits immunized with the electrophoretically homogenous proteins. IgG was purified from a pool of heat-inactivated antisera. After purification, several of the antibodies that cross-reacted with related forms of cytochrome P450 (i.e. polyspecific antibodies) were immunoabsorbed extensively to generate monospecific antibodies. Polyclonal (polyspecific) antisera against rat cytochrome P450 1A1, as well as a mixture of four monoclonal antibodies against rat cytochrome P450 1A1, were provided by Dr. Paul Thomas, Rutgers University.

Gel electrophoresis and immunoblots. SDS–PAGE was performed essentially as described by Laemmli

Table 1. Age, weight and tissue cytochrome P450 content for individual polar bears*

	Bear A	Bear B	Bear C	Bear D
Sex	M	M	M	M
Age (year)†	5	9	22	11
Body weight (kg)	167	320	310	358
Protein yield of hepatic microsomes (mg/g liver)	17.5	10.8	10.9	11.6
Cytochrome P450 content (nmol/g liver)	18.4	15.3	17.9	14.3
Cytochrome P450 content of hepatic microsomes (nmol/mg protein)	1.05	1.42	1.64	1.23
Protein yield of lung microsomes (mg/g lung)	1.21	0.76	—	1.01
Cytochrome P450 content of lung microsomes (nmol/mg protein)	ND‡	ND	ND	ND

* Polar bears were arbitrarily designated A–D.
† Age was determined by counting the *cementum annuli* on an extracted vestigial premolar tooth [43].
‡ Indicates that no measurable cytochrome P450 spectral peak was present.

Table 2. Catalytic activities of hepatic microsomes prepared from individual polar bears

Activity*	Bear A	Bear B	Bear C	Bear D
Aminopyrine *N*-demethylase (nmol HCHO formed/min/mg protein)	7.27	8.62	10.00	8.41
Benzphetamine *N*-demethylase (nmol HCHO formed/min/mg protein)	2.97	3.24	3.61	3.60
Ethylmorphine *N*-demethylase (nmol HCHO formed/min/mg protein)	9.55	10.42	10.68	9.10
p-Nitrophenol hydroxylase (nmol 4-nitrocatechol formed/min/mg protein)	1.10	1.15	1.47	1.23

* Results are the averages of duplicate determinations.

[40] in a 7.5% acrylamide separating gel that was 0.75 mm thick and 12.5 cm long. Proteins resolved on SDS–PAGE were transferred electrophoretically to nitrocellulose and probed with antibodies as described [41, 42]. Blots were incubated with primary antibodies at the concentrations specified on each figure (see Results). Bound primary antibody was located using horseradish peroxidase-conjugated goat anti-IgG second antibody. Immunoreactive proteins were detected subsequently by reaction of peroxidase with a substrate solution of either 4-chloro-1-naphthol (0.018%) and hydrogen peroxide (0.018%) or 3,3′-diaminobenzidine (0.025%), nickel chloride (0.02%) and hydrogen peroxide (0.006%), in phosphate-buffered saline, pH 7.4.

RESULTS

Cytochrome P450 content. Table 1 lists the values of the microsomal protein yield and cytochrome P450 content for microsomes prepared from adult, male polar bears. The total cytochrome P450 content per gram of liver was similar among the four bears, but the specific content varied from a value of 1 for bear A to approximately 1.6 nmol/mg microsomal protein for bear C. However, the cytochrome P450 content may be underestimated due to the presence of hemoglobin contamination in all four hepatic preparations. Lung microsomes from the four polar bears contained no spectrally detectable cytochrome P450, but a large peak at approximately 420 nm was present in all four lung samples. Carbon monoxide difference spectra obtained in the absence of sodium dithionite indicated the presence of large amounts of contaminating hemoglobin in these preparations.

Catalytic activities. Aminopyrine *N*-demethylase, benzphetamine *N*-demethylase, ethylmorphine *N*-demethylase and *p*-nitrophenol hydroxylase activities were measured in hepatic microsomes from individual polar bears and are reported in Table 2. Catalytic activities were not determined in lung microsomes because of the absence of a cytochrome P450 spectral peak in those preparations. Hepatic monooxygenase activities measured with each substrate were very similar among all four male bears, and no differences due to age or body weight were evident. Among the substrates, the highest activities were observed with aminopyrine and ethylmorphine and the lowest activity was seen with *p*-nitrophenol.

The capacity of polar bear hepatic microsomes to metabolize testosterone was also determined. The identity of the metabolites and their rates of formation are shown in Table 3. The primary oxidative metabolite was 2β-hydroxytestosterone, followed by 6β-hydroxytestosterone, androstenedione and, to a lesser extent, 16α-hydroxytestosterone. Minor pathways of testosterone oxidation include formation of 2α-, 7α- and 16β-hydroxytestosterone. Five other minor metabolites were present but could not be quantitated as we lacked the appropriate authentic standards. Comparison of the peak height and areas of the minor metabolites with those for which authentic standards were available indicated that the rates of formation of the unidentified metabolites were similar to those of 7α- and 16β-hydroxytestosterone. Testosterone hydroxylase activities were comparable among all four bears, and no major differences attributable to age or body weight were evident.

Electrophoretic profile. Figure 1 shows a Coomassie-blue stained polyacrylamide gel electro-

Table 3. Testosterone hydroxylase activities of hepatic microsomes from individual polar bears

	Testosterone metabolites*						
	2α	2β	6β	7α	16α	16β	Androstenedione
	(pmol metabolite formed/min/mg protein)						
Bear A	167	3495	1797	180	379	147	1179
Bear B	169	4329	2245	221	474	192	1189
Bear C	219	3816	1997	193	630	216	1159
Bear D	151	3280	1814	171	677	141	1192

Amount of each metabolite formed was calculated as described in Materials and Methods with the exception of androstenedione, for which the ratio of the peak height, instead of the peak area, was used to mitigate the effect of rising baseline at increased retention times.

* Results are the averages of duplicate determinations.

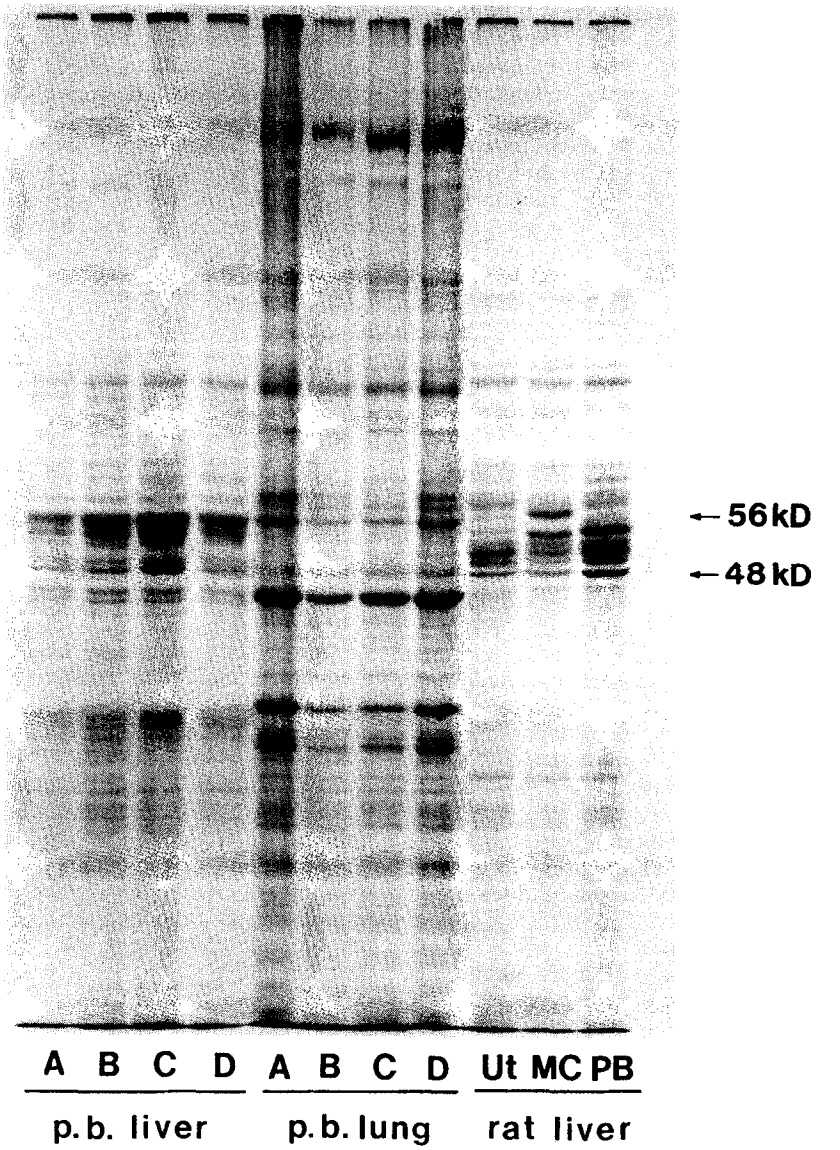

Fig. 1. Polyacrylamide gel electrophoretogram of polar bear (p.b.) liver, polar bear lung and rat liver microsomal samples. Lane designations A through D represent polar bears A through D. The abbreviations Ut, MC, and PB refer to untreated, 3-methylcholanthrene-treated, and phenobarbital-treated, respectively. Microsomal samples were applied at a final concentration of 10 μg of microsomal protein per lane. Proteins were stained with Coomassie Blue R-250.

phoretogram of polar bear liver and lung microsomes. Rat hepatic microsomes are included for purposes of comparison. Examination of the electrophoretic pattern of protein-stained bands revealed multiple proteins with apparent molecular weights of 50,000 (see arrows) in the lanes containing polar bear hepatic microsomes and fewer such proteins in the lanes containing polar bear lung microsomes. From the electrophoretogram, it appeared that numerous forms of cytochrome P450 are expressed in polar bear liver and that several of these proteins are not expressed in the lung. Moreover, the electrophoretic mobilities of the polar bear hepatic proteins were not the same as those from untreated, MC-treated or PB-treated rats.

Immunochemical reactivity. Polar bear hepatic and lung microsomal samples were analyzed on immunoblots probed with antibodies generated against various forms of rat cytochrome P450. The specificity of each antibody had been assessed previously using both enzyme-linked immunosorbent and immunoblot assays with purified rat cytochrome P450 isozymes and with different rat liver microsomal preparations (results not shown). Liver microsomes from untreated rats and rats pretreated with MC and PB are included on the blots containing the polar bear samples for purposes of comparison.

Figure 2 illustrates immunoblot results using two different anti-rat cytochrome P450 1A1 antibodies, as well as anti-rat cytochrome P450 2B1 IgG. As shown in Fig. 2A, a mixture of monoclonal antibodies against rat cytochrome P450 1A1 recognized a single protein band in liver microsomes from all four polar bears, but no reaction was observed with polar bear lung microsomes at the protein concentrations used. A single darkly stained band, corresponding to cytochrome P450 1A1, was detected in the lane containing hepatic microsomes from MC-treated rats but not in the lanes containing microsomes from untreated or PB-treated rats. The immunoblot indicates that polar bears have a hepatic cytochrome P450 protein that is immunochemically related to rat cytochrome P450 1A1. The bear protein migrated faster on the polyacrylamide gel than rat cytochrome P450 1A1, suggestive of a smaller molecular weight. Results obtained with the monoclonal antibody mixture were corroborated using polyclonal rabbit anti-cytochrome P450 1A1 sera, a relatively crude antibody preparation raised against rat cytochrome P450 1A1 that cross-reacts strongly with cytochrome P450 1A2 (see Fig. 2B). The antisera recognized two proteins in polar bear microsomes, did not react with the lung microsomes, and detected several bands in rat liver microsomes. The intensely stained band observed in the lane containing microsomes from MC-treated rats represents cytochrome P450 1A1, while the band immediately below is cytochrome P450 1A2. Together with the preceding blot, the results imply that polar bear liver contains proteins that are immunochemically related to rat cytochromes P450 1A1 and P450 1A2.

As shown in Fig. 2C, antibody against rat cytochrome P450 2B1 reacted strongly with hepatic microsomes from polar bears. In addition, a very faint band with identical electrophoretic mobility was observed in all four lung samples. Three immunostained protein bands, corresponding to cytochromes P450 2B2 (upper band), cytochrome P450 2B1 (middle band) and a related protein (lower band), possibly cytochrome P450 2B3, are apparent in liver microsomes prepared from untreated rats. As expected, the intensity of the middle band (cytochrome P450 2B1) increased dramatically after pretreatment of rats with PB. The immunoblot demonstrates that an immunoreactive analogue of the rat cytochrome P450 2B subfamily is expressed in both polar bear liver and lung.

To determine if cytochrome P450 proteins belonging to the cytochrome P450 2C subfamily are found in polar bears, an immunoblot was probed with polyspecific anti-rat cytochrome P450 2C11 IgG (Fig. 3A), an antibody that cross-reacts with rat cytochromes P450 2C6, 2C7, 2C12 and 2C13, in addition to the antigen of immunization. Subsequent blots were probed with antibodies specific to individual members of the subfamily (Fig. 3, B and C). The polyspecific antibody recognized several protein bands in hepatic microsomes prepared from both polar bears and rats. In contrast, no protein bands were detected in any of the polar bear samples with monospecific anti-rat cytochrome P450 2C13 IgG (Fig. 3C) or anti-rat cytochrome P450 2C7 IgG (results not shown). On the blot probed with monospecific anti-rat cytochrome P450 2C11 IgG (Fig. 3B), a faint band was barely visible in the lanes containing polar bear liver microsomes, while a dark band was apparent in the lanes containing rat liver microsomes. Unfortunately, this antibody preparation was not completely monospecific, as demonstrated by the presence of a second protein band with microsomes from PB-treated rats.

In Fig. 4A, rabbit polyspecific anti-rat cytochrome P450 3A1 IgG was used as the primary antibody. We have determined previously that this antibody reacts with both cytochromes P450 3A1 and 3A2 and several other cytochrome P450 isozymes. As shown in Fig. 4A, three broad protein bands were detected in polar bear liver microsomes and a few weaker bands were present in lung microsomes in the cytochrome P450 molecular weight region. Several bands were evident in the lanes containing hepatic microsomes prepared from both untreated and treated adult male rats. We then passed the antibody through columns containing immobilized hepatic microsomes prepared from untreated and isoniazid-treated rats in an attempt to remove the cross-reaction with cytochrome P450 3A2, as well as with other structurally unrelated isozymes. The back-absorbed antibody, although not completely monospecific, gave a single weakly stained band with liver samples from the polar bears (Fig. 4B). The diffuse upper and lower bands were no longer visible. In addition, no reaction was detected with polar bear lung microsomes. A single protein band of varying staining intensity was observed with rat liver microsomes.

An antibody to rat epoxide hydrolase was employed to examine polar bear microsomes for the presence of an immunochemically related form of this enzyme. The resulting immunoblot (Fig. 4C) shows a stained band in polar bear hepatic microsomes, but no reaction was observed with polar

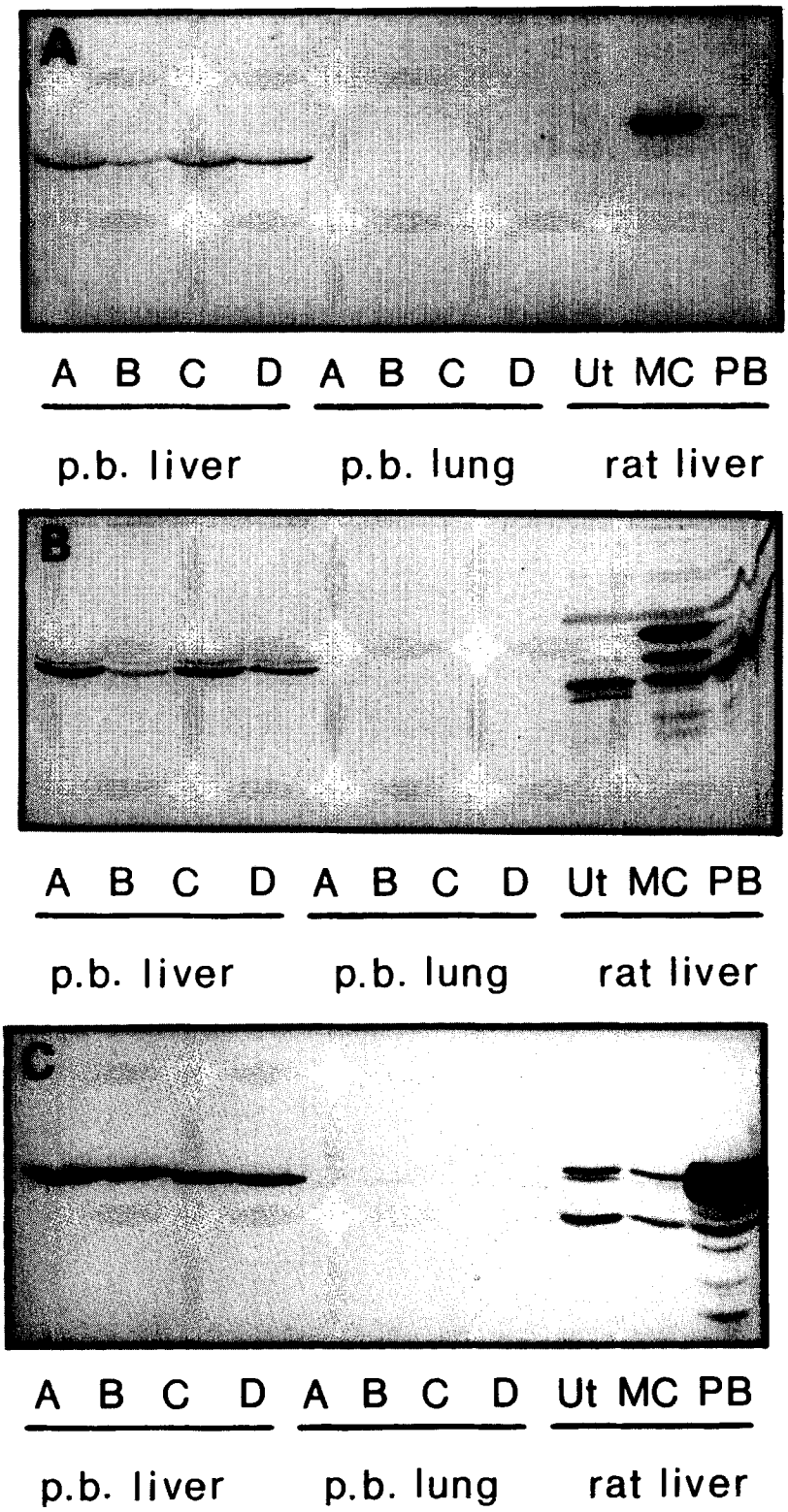

Fig. 2. Immunoblots of polar bear (p.b.) liver and lung microsomes probed with monoclonal mouse anti-rat cytochrome P450 1A1 IgG mixture at a concentration of 1 μg IgG/mL (panel A), rabbit anti-rat cytochrome P450 1A1 sera at a 1:1000 dilution (panel B), and rabbit anti-rat cytochrome P450 2B1 IgG at 3 μg IgG/mL (panel C). Polar bear microsomal samples were applied to the gel at a final concentration of 20 μg of microsomal protein per lane for immunoblots A and B, and at a final concentration of 10 μg per lane for immunoblot C. All rat liver microsomal samples were applied to the gel at a final concentration of 10 μg of microsomal protein per lane. Refer to the legend of Fig. 1 for the definition of lane designations.

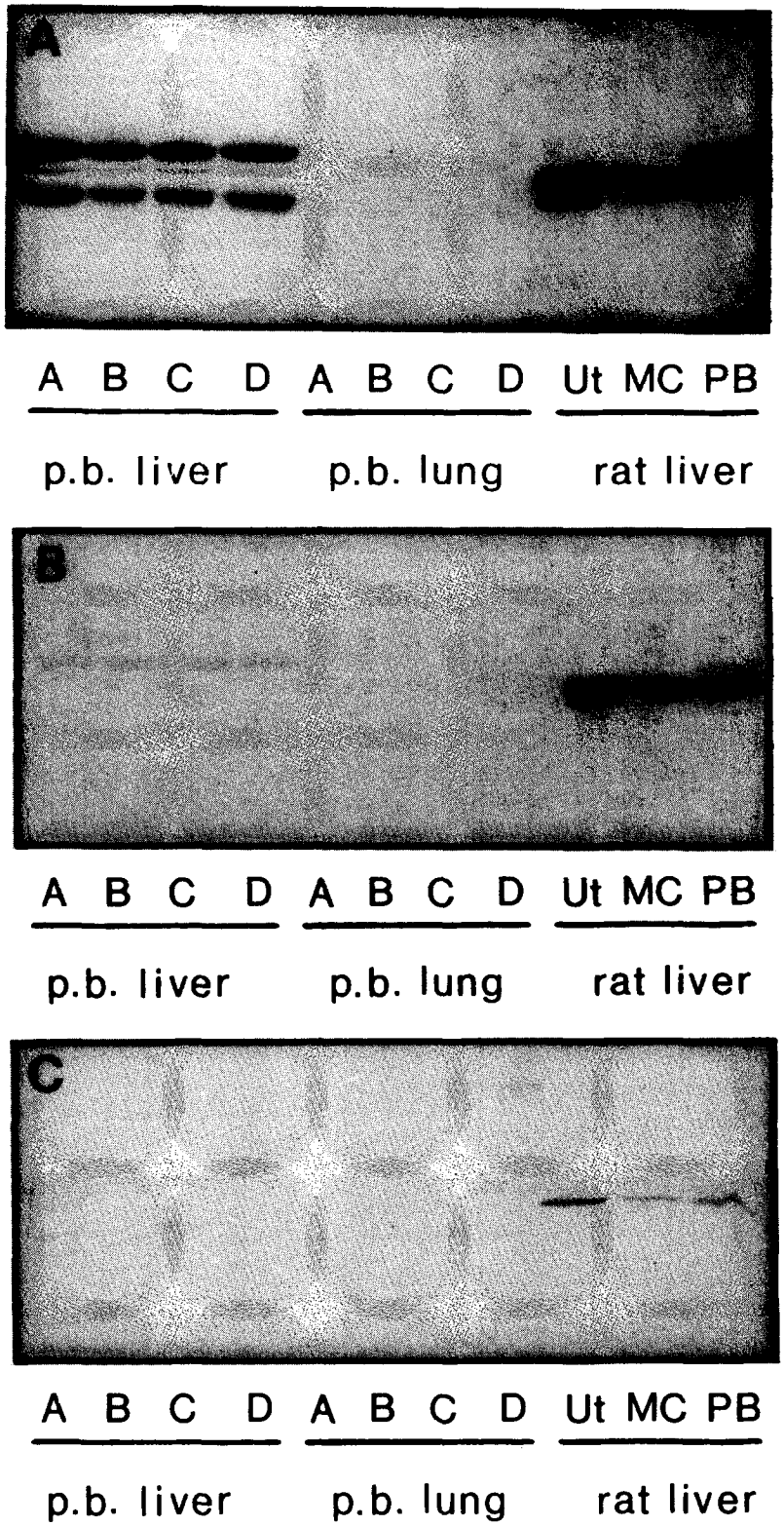

Fig. 3. Immunoblot of polar bear (p.b.) liver and lung microsomes probed with polyspecific rabbit anti-rat cytochrome P450 2C11 IgG at a concentration of 40 μg IgG/mL (panel A), monospecific rabbit anti-rat cytochrome P450 2C11 IgG at 17 μg IgG/mL (panel B), and monospecific rabbit anti-rat cytochrome P450 2C13 IgG at 50 μg IgG/mL (panel C). Polar bear microsomal samples were applied to the gel at a final concentration of 20 μg of microsomal protein per lane for immunoblots A and C, and at a final concentration of 10 μg of microsomal protein per lane for immunoblot B. All rat liver microsomes were applied to the gel at a final concentration of 10 μg of microsomal protein per lane. Refer to the legend of Fig. 1 for the definition of lane designations.

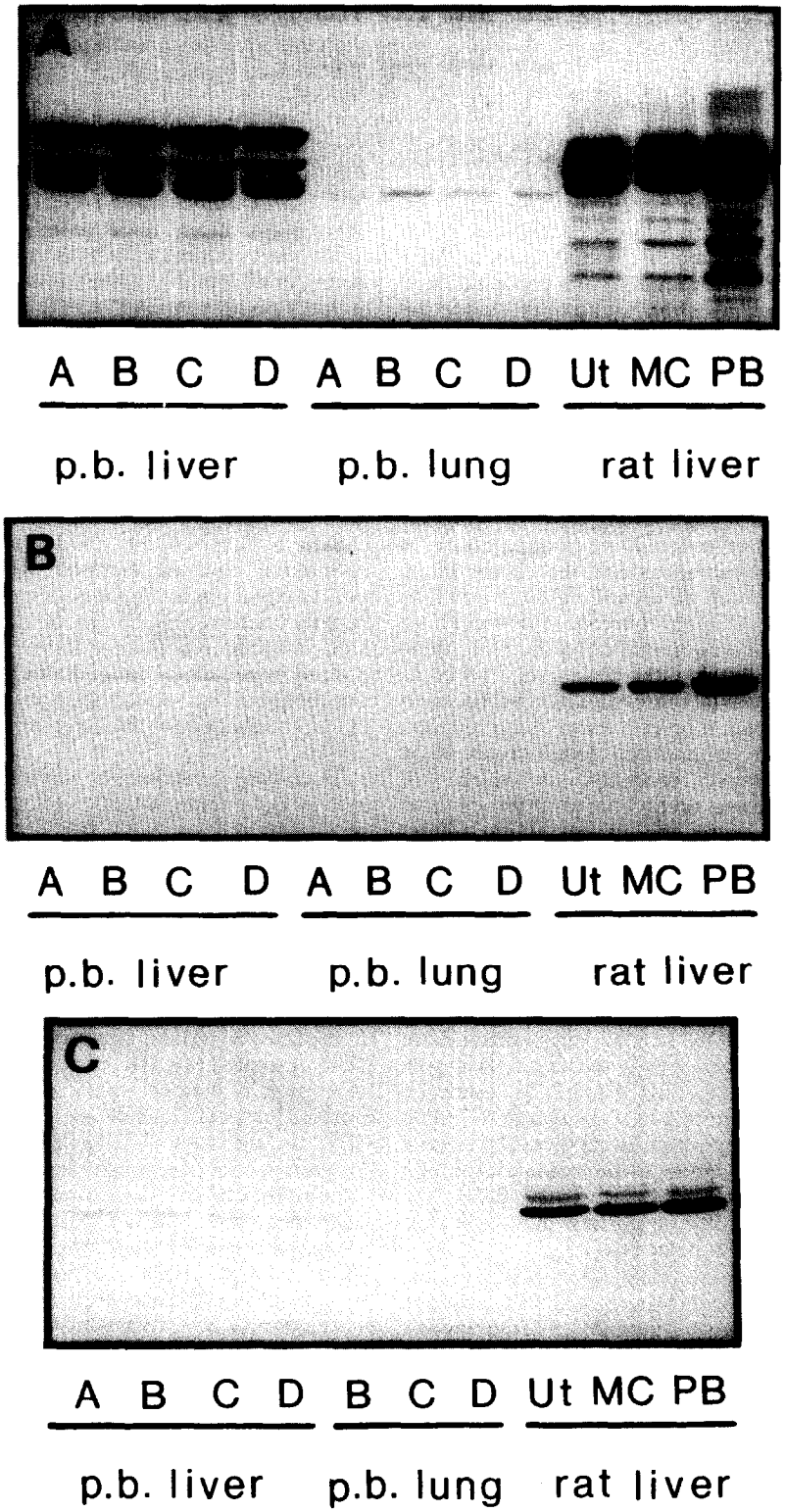

Fig. 4. Immunoblots of polar bear (p.b.) liver and lung microsomes probed with rabbit anti-rat cytochrome P450 3A1 IgG at a concentration of 50 μg IgG/mL (panel A), anti-rat cytochrome P450 3A1 IgG, after back-absorption, at 50 μg IgG/mL (panel B), and rabbit anti-rat epoxide hydrolase IgG at 50 μg IgG/mL (panel C). Polar bear microsomal samples were applied to the gel at a final concentration of 10 μg of microsomal protein per lane for immunoblots A and B, and at a final concentration of 20 μg of microsomal protein per lane for immunoblot C. All rat liver microsomes were applied to the gel at a final protein concentration of 10 μg of microsomal protein per lane. Note that the lung microsomal preparation for bear A is not included on the final immunoblot (panel C) because the supply of this sample was depleted. Refer to the legend of Fig. 1 for the definition of lane designations.

bear lung microsomes at the protein concentrations used.

DISCUSSION

In the present study, we set out to characterize microsomal cytochrome P450 enzymes in the polar bear by examining both monooxygenase activities and the immunochemical relatedness between polar bear and rat cytochromes P450. Cytochrome P450 content has been used frequently as a crude measure of the xenobiotic oxidation capacity of an animal and is a major factor in determining xenobiotic half-life. The hepatic cytochrome P450 content of the four male polar bears was similar to cytochrome P450 content measured in rats, but higher than levels reported for marine mammals [44–47]. Unfortunately, there was no measurable cytochrome P450 in polar bear lung microsomes due, in part, to the low microsomal yield from this tissue and the presence of contaminating hemoglobin, and as a result this tissue was not included in the measurement of enzymatic activities. Xenobiotic substrates including aminopyrine, benzphetamine, ethylmorphine and *p*-nitrophenol were metabolized by polar bear hepatic microsomes at rates that were similar to those obtained in our laboratory for liver microsomes from untreated adult rats.* Polar bear hepatic microsomes also metabolized testosterone to a variety of oxidative products, of which 2β-hydroxytestosterone, 6β-hydroxytestosterone and androstenedione were the primary metabolites. Although testosterone hydroxylase activity was considerably greater in the polar bear, the pattern of testosterone metabolites generally resembled that obtained with goat hepatic microsomes [48], and goat, cow and sheep hepatocytes in culture [49]. In contrast, 2α-, 6β- and 16α-hydroxytestosterones, together with androstenedione, are the major metabolites formed by hepatic microsomes from untreated adult male rats [39]. In the rat, oxidation of testosterone at the 2β position is catalyzed principally by cytochromes P450 3A1 and 3A2, while oxidation at the 6β position is catalyzed by several cytochromes P450, and androstenedione formation is catalyzed mainly by cytochromes P450 2B1 and 2C11 [1, 2, 39, 50], suggesting that analogous isozymes occur in the polar bear.

Additional and more definitive evidence for the presence of specific cytochrome P450 orthologues in polar bear tissue can be provided by immunoblot analysis. Antibodies prepared against purified rat cytochrome P450 isozymes can be used to identify and characterize the cytochrome P450 composition in polar bear. The feasibility of this approach has been demonstrated in several studies [29–31]. Antibodies to rat cytochromes P450 have been shown to react with analogous forms from diverse mammalian species [51–53], fish [30, 54] and birds [29, 55].

On the basis of the immunoblot results, we conclude that polar bear liver contains cytochrome P450 isozymes that are immunochemically related to rat cytochromes P450 1A1, 1A2, 2B1 and 3A1. Several cytochrome P450 proteins that are related to the rat cytochrome P450 2C subfamily, but that do not correspond to cytochromes P450 2C7, 2C11 or 2C13 are also present. Although a weak reaction between monospecific anti-rat cytochrome P450 2C11 IgG (see Fig. 3B) and polar bear hepatic microsomes were observed, the poor staining intensity of the resulting band relative to the dark band observed with rat liver microsomes, together with the fact that the antibody was not completely monospecific, strongly suggest that the immunoreactive protein was not a polar bear homologue of rat cytochrome P450 2C11 but a related isozyme. In addition, a protein that is recognized by antibody to rat epoxide hydrolase was detected in polar bear hepatic microsomes. Of the isozymes identified in liver, only the cytochrome P450 2B1 homologue is present in polar bear lung, although we cannot rule out the possibility that other isozymes were present but at levels below the limit of detection of our assays.

There was considerable variability noted in the intensity of staining with the various antibodies. This variability reflects both the degree of immunochemical relatedness between rat and polar bear isozymes and the tissue levels of the isozymes. On the blots probed with monoclonal antibody to cytochrome P450 1A1, anti-cytochrome P450 1A1 sera and anti-cytochrome P450 2B1 IgG (Fig. 2), the intensity of the stained protein bands in the lanes containing polar bear liver was much greater than in the lanes containing hepatic microsomes prepared from untreated rats. The most likely explanation for this result is that hepatic levels of the polar bear homologues of cytochromes P450 1A1, 1A2 and 2B1 are elevated. Unlike genetically equivalent laboratory animals maintained under controlled conditions, wild animals are genetically more diverse, and their diet, disease history, and xenobiotic exposure are often unknown. Induction of one or more cytochromes P450 in these animals is possible as a result of exposure to chemical contaminants in the environment. Organochlorine residues consisting of almost equal portions of chlordane, PCBs and related compounds at levels ranging from 5 to 7 μg/g of lipid in liver were reported previously for polar bears in the Canadian arctic and subarctic [16]. Currently, we are analyzing adipose and liver tissue from the polar bears for organochlorine content as part of a larger study examining the relationship between immunoreactive hepatic cytochrome P450 1A1, 1A2 and 2B1 protein levels, the rates of ethoxyresorufin and pentoxyresorufin *O*-dealkylase activities, and organochlorine tissue concentrations in sixteen polar bears in order to determine if contaminant exposure in this species is reflected in the hepatic cytochrome P450 profile. Despite the high level of immunoreactive cytochrome

* For purposes of comparison, values of aminopyrine *N*-demethylase, benzphetamine *N*-demethylase, ethylmorphine *N*-demethylase and *p*-nitrophenol hydroxylase activities determined in our laboratory with hepatic microsomes from untreated adult male rats were 6.89 ± 0.47, 5.30 ± 0.15 and 9.95 ± 0.36 nmol HCHO formed/min/mg protein, and 1.09 ± 0.10 nmol 4-nitrocatechol formed/min/mg protein, respectively (mean ± SEM, N = 6–8).

P450 2B1 protein detected in the polar bear livers, aminopyrine *N*-demethylase and benzphetamine *N*-demethylase activities, which correlate with hepatic cytochrome P450 2B1 levels in induced rats, did not appear to be induced in the polar bears. A possible explanation for these discrepant data is that the polar bear cytochrome P450 2B1 homologue does not catalyze the same enzyme activities as rat cytochrome P450 2B1. Alternately, the K_m of the cytochrome P450 2B1 homologue in polar bear may be different from that in rat.

A confounding factor in the present study is that the bears were immobilized with Telazol®. We have not found any reports in the literature regarding the inducing properties of Telazol® or its ingredients, but a study is underway in our laboratory to determine if pretreatment with Telazol® has any effect on hepatic levels of cytochromes P450 1A or 2B in rats.

In conclusion, the present study indicates the presence of cytochrome P450 1A1, 1A2, 2B1, and 3A1 homologues and forms related to the cytochrome P450 2C subfamily, as well as epoxide hydrolase in polar bear liver microsomes. To the best of our knowledge this is the first study that has investigated and attempted to characterize ursine cytochromes P450. Additional studies are needed to determine the influence of halogenated compounds on cytochrome P450 levels, and in particular on cytochrome P450 1A levels, in polar bear and to determine whether induction, as measured by immunoquantitation, can be used as a sensitive biological indicator of chemical exposure in the arctic marine ecosystem.

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