level exposure of possibly hundreds of environmental inducers.

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Changes in hepatic drug metabolism in alloxan-diabetic male rabbits

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In rats, it has been clearly demonstrated that chemically-induced diabetes mellitus produces changes in hepatic microsomal drug metabolism. The activities of sex-dependent enzymes (aminopyrine demethylase, benzo[*a*]pyrene hydroxylase) decrease while those of sex-independent enzymes (aniline hydroxylase) increase following the induction of diabetes in male rats [1–5]. In female rats, by contrast, diabetes causes a more generalized increase in enzyme activities. The effects of diabetes on hepatic monooxygenases can be prevented or reversed by insulin administration to diabetic animals. Some of the changes in drug-metabolizing activities caused by diabetes may be attributable to alterations in the cytochrome P-450 composition of hepatic microsomes. Past and Cook [6] have found far greater amounts of a cytochrome P-450 isozyme, which catalyzes aniline hydroxylation, in microsomal preparations from diabetic than from control rats. Recent studies [7] indicate that the spin state of hepatic cytochromes P-450 may also be modified in diabetic animals. Thus, changes in the ratio of cytochrome P-450 isozymes, as well as in the functional properties of cytochromes P-450, may account for the changes in monooxygenase activities caused by diabetes.

In contrast to the large number of investigations done with rats, there have been relatively few reports on the relationship between diabetes and hepatic drug metabolism in other species. Thus, it is not clear whether the observations made in rats are generally applicable or species-specific. Diabetes seems to have different effects on hepatic monooxygneases in guinea pigs than in rats. In spontaneously diabetic male guinea pigs, there is a decrease in aniline hydroxylase activity and no change in the rate of aminopyrine demethylation [8]. Diabetes has no effect on drug metabolism in female guinea pigs [8]. In untreated human diabetics, the *in vivo* half-lives of acetophenetidin and antipyrine are prolonged, suggesting changes in hepatic drug metabolism [9, 10]. The changes are reversed by insulin treatment. In rabbits, the urinary excretion of unaltered acetophenetidin is greater and metabolite excretion lower in diabetics than in controls following intraperitoneal administration of the drug [11]. Similarly, metabolism of acetophenetidin *in vitro* is lower in microsomal preparations from the diabetic rabbits than those from the controls but the metabolism of other substrates was not evaluated. Because of the relative paucity of data concerning the impact of diabetes mellitus on hepatic drug metabolism in species other than rats, we have pursued the observations of Dajani and Kayyali [11] and studied the effects of diabetes on various hepatic microsomal monooxygenases in rabbits. The results presented in this communication further indicate that the qualitative changes in drug-metabolizing enzymes caused by diabetes are species-specific.

Methods

Adult (1.5 to 2.5 kg) male New Zealand White rabbits obtained from Green Meadows Rabbitry (Murraysville, PA) were used for all experiments. Rabbits were maintained under standard conditions of light (6:00 a.m.–6:00 p.m.) and temperature (22°), and they received food and water *ad lib*. Animals were made diabetic following an 18–24 hr fast, by an i.v. infusion of alloxan (100 mg/kg) in 0.02 M citrate saline via the marginal ear vein. Age-matched controls received an infusion of vehicle alone. The induction of diabetes was confirmed by analysis of urine samples with Keto-Diastix (Ames). Rabbits were killed 1 or 2 months after the induction of diabetes (between 8:30 and 9:30 a.m.) by a blow to the head followed by exsanguination. Blood samples were taken, the serum was separated, and serum glucose concentrations were determined using the ABTS method of Bergmeyer and Bernt [12]. Livers were rapidly removed at the time of sacrifice and placed in ice-cold 1.15% KCl containing 0.05 M Tris–HCl (pH 7.4). Tissues were homogenized and microsomes were

obtained by differential centrifugation as previously described [13]. Microsomal preparations were washed once and resuspended in KCl–Tris buffer for use in experiments.

Aniline hydroxylase activity was assayed as the rate of *p*-aminophenol production by hepatic microsomes as described previously [14, 15]. Benzo[*a*]pyrene hydroxylation was determined by the fluorometric method of Nebert and Gelboin [16]. Quinine sulfate was calibrated against authentic 3-OH-benzo[*a*]pyrene and routinely used as the fluorescence standard. Benzphetamine N-demethylation was assayed as the amount of formaldehyde formed using the method of Nash [17], as previously described [13]. For all enzyme assays, conditions were established to ensure linearity of product formation with respect to protein concentrations and incubation times.

Cytochrome P-450 was measured as the dithionite-reduced CO complex as described by Omura and Sato [18] with an Aminco DW-2a recording spectrophotometer. NADPH–cytochrome *c* reductase activity was assayed by the method of Phillips and Langdon [19]. NADPH–cytochrome P-450 reductase activity was determined with an Aminco DW-2a recording spectrophotometer in the dual wavelength (450–490 nm) mode, essentially as described by Gigon *et al.* [20] and modified by Jansson and Schenkman [21]. The reaction was run at 22°. Microsomal protein was assayed by the method of Lowry *et al.* [22]. Data were analyzed by Student's *t*-test with a probability level of $P < 0.05$ considered significant.

Results and discussion

Blood glucose levels were elevated significantly at both 1 and 2 months after the induction of diabetes (Table 1). Body and liver weights were lower in the diabetics than in controls, but all animals appeared to be in good health at the time of sacrifice. Hepatic microsomal protein concentrations were significantly higher in the 1-month diabetics than in the corresponding controls, but at 2 months values were similar in the controls and diabetics.

Table 1. Effects of alloxan-induced diabetes mellitus on body and liver weights, blood glucose levels, and microsomal protein content in New Zealand White rabbits

	Body wt (kg)	Blood glucose (mg/100 ml)	Liver wt (g)	Hepatic microsomal protein (mg/g liver)
One-month control	3.06 ± 0.09	83.9 ± 10.6	105.01 ± 8.5	33.3 ± 1.5
One-month diabetic	2.51 ± 0.08*	331.5 ± 46.6*	76.91 ± 5.3*	40.2 ± 2.2*
Two-month control	3.64 ± 0.10	114.3 ± 6.1	106.21 ± 2.2	32.2 ± 1.9
Two-month diabetic	3.25 ± 0.13*	478.8 ± 20.6*	92.84 ± 4.2*	35.6 ± 1.6

Data represent means ± S.E.M. of five to ten observations.
* $P < 0.05$ (vs corresponding controls).

Aniline hydroxylase activity was significantly higher in diabetic rabbits than in the controls at both 1 and 2 months (Table 2). The rate of metabolism of the type II substrate was greater in diabetics regardless of whether activity was expressed per gram of tissue or per milligram of microsomal protein. The increase in aniline hydroxylase activity following the induction of diabetes was similar to that previously noted in rats [1–5], but opposite to the observations made in diabetic guinea pigs [8]. In contrast to the effects of diabetes on aniline metabolism, there was little change in the rates of metabolism of the type I substrates, benzo[*a*]pyrene and benzphetamine (Table 2). In the 1-month diabetics, benzo[*a*]pyrene hydroxylase activity, when expressed per gram of liver, was elevated significantly. However, when corrected for the differences in microsomal protein concentrations, enzyme activities were similar in the controls and diabetics (Table 2). The rates of benzo-[*a*]pyrene hydroxylation at 2 months and of benzphetamine demethylation at both 1 and 2 months after the induction of diabetes did not differ in the controls and diabetics, regardless of the mode of expression of enzyme activities. These results differ from those obtained in rats in which diabetes causes a decrease in the microsomal metabolism of type I xenobiotic substrates in males and an increase or no change in females [1–5].

Diabetes had no effect on cytochrome P-450 concentrations (per mg protein) in rabbit liver microsomes (Table 2). At both 1 and 2 months, the concentrations of cytochrome P-450, when expressed per mg of protein, were similar in the control and diabetic animals. However, when expressed per g liver, cytochrome P-450 concentrations were greater in the diabetics than in the controls at 1 month but not at 2 months. NADPH–cytochrome *c* reductase activity was unaffected by diabetes at either 1 or 2 months (Table 2). However, NADPH–cytochome P-450 reductase activity, determined only at 1 month after the induction of diabetes, was substantially greater in the diabetic animals than in the controls (Table 2). The mechanism(s) responsible for the dramatic increase in activity is not known. However, Rouer and coworkers [7] have reported recently that diabetes causes a change in the spin state of hepatic microsomal cytochrome(s) P-450 in rats and in mice. Since the spin state of cytochrome P-450 influences its rate of reduction by NADPH–cytochrome P-450 reductase, such a change may account for the increase in activity we have observed. That possibility will be considered in future experiments.

Our observations provide further evidence that diabetes mellitus causes changes in hepatic microsomal drug metabolism. However, the nature of the changes appears to be species dependent. Even among the limited number of small mammalian species studied, the specific enzymatic changes vary from species to species. Thus, extrapolation of specific effects to higher species does not seem warranted although diabetes appears to have some effects on drug metabolism in all species studied. Previous investigations have implicated changes in cytochrome(s) P-450 in the effects of diabetes on hepatic drug metabolism [6, 7]. The effects of diabetes on the reduction of cytochrome P-450 presented in this report lend further support to that hypothesis. However, additional investigations are clearly needed before definitive mechanisms can be established.

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Table 2. Effects of alloxan-induced diabetes mellitus on hepatic microsomal metabolism in New Zealand White rabbits

	One-month control	One-month diabetic	Two-month control	Two-month diabetic
Aniline hydroxylase				
(nmoles/min × g liver)	11.5 ± 1.4	22.7 ± 1.6*	13.4 ± 1.7	20.9 ± 2.0*
(nmoles/min × mg protein)	0.41 ± 0.04	0.58 ± 0.04*	0.43 ± 0.06	0.60 ± 0.06*
Benzo[*a*]pyrene hydroxylase				
(nmoles/min × g liver)	5.4 ± 0.4	7.1 ± 0.5*	6.1 ± 0.7	5.9 ± 0.5
(nmoles/min × mg protein)	0.15 ± 0.01	0.16 ± 0.01	0.17 ± 0.02	0.16 ± 0.01
Benzphetamine demethylase				
(nmoles/min × g liver)	283.4 ± 22.5	331.5 ± 10.6	267.6 ± 15.6	310.8 ± 22.4
(nmoles/min × mg protein)	7.8 ± 0.3	7.6 ± 0.3	8.4 ± 0.6	8.5 ± 0.6
Cytochrome P-450				
(nmoles/g liver)	28.7 ± 1.5	34.0 ± 1.1*	35.7 ± 3.1	36.2 ± 2.1
(nmoles/mg protein)	0.91 ± 0.07	0.92 ± 0.06	1.03 ± 0.12	1.10 ± 0.08
NADPH-cytochrome *c* reductase				
(nmoles/min × g liver)	652 ± 68	741 ± 70	637 ± 61	683 ± 73
(nmoles/min × mg protein)	17.8 ± 1.6	18.1 ± 1.7	16.9 ± 1.5	17.5 ± 1.8
NADPH–cytochrome P-450 reductase				
(nmoles/min × g liver)	6.4 ± 1.3	27.9 ± 2.7*	ND†	ND
(nmoles/min × mg protein)	0.17 ± 0.03	0.64 ± 0.09*	ND	ND

Data represent means ± S.E.M. of six to ten observations.
* $P < 0.05$ (vs corresponding controls).
† Not determined.

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Boron uptake in melanoma, cerebrum and blood from $Na_2B_{12}H_{11}SH$ and $Na_4B_{24}H_{22}S_2$ administered to mice

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The nuclear reaction $^{10}B(n, \alpha\gamma)^7Li$ [1] has been used in boron neutron capture therapy (BNCT*) [2] to cure malignant gliosarcomas implanted in the hind legs of mice [3, 4] and spontaneous malignant melanomas in pigs [5]. The sulfhydryl borane monomer $[B_{12}H_{11}SH]^{2-}$ (BSH) [6, 7] is used as a ^{10}B carrier for BNCT of malignant human brain tumors [8, 9]. It has been suggested that its disulfide dimer, $[B_{24}H_{22}S_2]^{4-}$ (BSSB) [10, 11], and its disulfide monoxide dimer [12] may also have favorable properties as carriers of ^{10}B for BNCT. We now report that Na_4BSSB yields higher tumor–blood and tumor–cerebrum boron concentration differences than does Na_2BSH when each is infused to deliver the same dose of boron (~200 μg B/g body wt) very slowly (~1 μg B/g body wt hr) into tumor-bearing mice. This observation may have implications for BNCT of human brain tumors such as malignant gliomas, which have an imperfect blood–brain barrier and which, therefore, are more accessible to Na_4BSSB or to Na_2BSH than are normal brain tissues.

Methods and results

Cs_4BSSB was synthesized by oxidation of Cs_2BSH (Callery Chemical Co., Callery, PA) using *o*-iodosobenzoic acid as described by Wellum *et al.* [11]. The sparsely soluble cesium salts of these two boranes were converted to their highly soluble sodium salts as follows. One hundred milligrams of the cesium salt was dissolved in 15 ml of warm water with stirring and then passed through an ion-exchange column (1.5 × 1.0 cm) containing 0.6 g of 100–200 mesh Dowex 50 W-X8. Recovery efficiency of the column, as judged by prompt gamma analysis of boron [13] in aliquots of column inflow and outflow, was >90%. X-ray fluorescence analysis [14] of the sodium salt of BSH failed to reveal residual cesium.

Thin-layer chromatography (TLC) with 3 M aqueous NH_4NO_3–acetonitrile (2:1) on DEAE cellulose plates (Brinkmann, Westbury, NY) [15] was used to detect contamination of Na_4BSSB ($R_f = 0.23$) by Na_2BSH ($R_f = 0.56$). The visible spectrum of the blue, non-degassed solution of 2.3×10^{-4} M Cs_4BSSB in the colourless solvent, 5.0×10^{-3} M trifluoroacetic acid in dimethylformamide, had one broad absorbance band which was maximum at 628 nm. The 628 nm absorbance of the solution decreased slowly with a half-life of ~66 days, much longer than that reported originally (~8 days) [11]. The initial absorbance of the solution, 0.71, was only two-thirds of the expected [11] absorbance.

ESR of the blue solution of Cs_4BSSB in the acidified dimethylformamide solvent was performed with a Varian E-line X-band spectrometer calibrated for field sweep with an aqueous solution of Fremy's salt, $K_2(SO_3)_2NO$, and for measurements of g values with a solution of perylene in concentrated sulfuric acid. A single peak was found at $g = 2.023$. The previously reported value was 2.019 [11]. The peak-to-peak width of the first derivative of the signal was 18.6 G. The signal width at half-maximum intensity was 26.0 G, more than the reported [11] value of 19.3 G. The 628 nm absorbance and the intensity of the ESR signal of the blue solution decreased with time, but a constant arithmetic ratio of these quantities to each other was maintained meanwhile. Other observations which show that Na_4BSSB was the principal solute in the indicated (Tables 1–3) mouse infusions were the exact correspondence of the principal infrared absorption bands of the cesium salt of our preparation with those of Cs_4BSSB [11] and the absence of significant contamination of our Na_4BSSB preparations by Na_2BSH when they were tested by TLC.

Aqueous solutions of Na_2BSH or Na_4BSSB were infused slowly intraperitoneally (Osmotic pump, model 2001, Alza Corp., Palo Alto, CA) or were injected intraperitoneally promptly into 15–20 g female BALB/cJ mice bearing subcutaneously implanted Harding–Passey (HP) melanomas [16].

All borane solutions loaded into osmotic pumps were in water except in mouse group L (Table 2) where 5.3×10^{-3} M sodium metabisulfite ($Na_2S_2O_5$) was added to retard oxidation of BSH. Mice were deeply anesthetized with ether and killed by exsanguination from the heart at the times specified in the tables. These osmotic pumps were designed to function for 219 ± 6 hr, but the precise duration of infusion in each mouse varied, either because the mouse was killed before the scheduled cessation of pump function or perhaps because some of the pumps were used several years after their manufacture and therefore pumped more slowly or more rapidly than expected. Focal hepatic necrosis and fibrosis which were observed in some mice bearing the rigid cylindrical (3.0 × 0.7 cm) osmotic pumps for 8–10 days may have been due to direct pressure on the liver or its vasculature.

Tissue and blood specimens in quartz test tubes, with sufficient H_2O added to bring each sample to a total weight of 1.00 g, were analyzed for boron by counting 478 keV gamma photons from the $^{10}B(n, \alpha\gamma)^7Li$ reaction during irradiation by slow neutrons [13]. The results of boron analyses of HP melanomas, whole blood and cerebra are shown in Tables 1–3.

Variations of boron doses to individual mice were due mainly to special experimental conditions. Mice were selected for infusion or injection on the basis of tumor size rather than body weight. Moreover, the volumes of individual

* Abbreviations: BNCT, boron neutron capture therapy; ESR, electron spin resonance; BSH, $(B_{12}H_{11}SH)^{2-}$; BSSB, $(B_{12}H_{11}S—SB_{12}H_{11})^{4-}$ or $(B_{24}H_{22}S_2)^{4-}$; and LET, linear energy transfer.