Influence of Age and Diet on the Induction of Microsomal Enzymes in the Mouse[1] (35387)

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(Introduced by J. M. Coon)

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The duration of action of a large number of drugs can be correlated with their rate of detoxication by specific liver enzymes. The activity of these enzymes is dependent on the age, dietary intake, and previous drug treatment of the animal (1–3). The rate of drug detoxication can be increased by treating animals with any of a variety of agents which induce microsomal drug-metabolizing enzymes. In a previous report, it was shown that the capacity of such agents to decrease hexobarbital sleeping time in mice was also dependent on the age and dietary status of the animal (4). The decrease in hexobarbital sleeping time was assumed to be due to an increase in microsomal mixed function oxidase activity. The activity of this enzyme system is related to the presence of cytochrome P-450 in the liver microsomes. In the present study it has been observed that the age and dietary status of the mouse not only influences the degree of enzyme induction but also may determine the type of cytochrome P-450 that is induced in the liver by these agents.

Material and Methods. Adult and 21-day-old weanling CD 1 male mice were kept in air-conditioned animal quarters and maintained on Purina laboratory chow. Food, but not water, was withheld for 48 hr from one group of adult mice prior to sacrifice. Weanling mice were maintained on isocaloric protein diets of either 8 or 27% casein for 7 days before the beginning of the experimental study.

The induction studies were accomplished by the daily intraperitoneal administration of equimolar doses (in a volume of 0.01 ml/g of body wt) of one of the following agents for 3 consecutive days: Na phenobarbital (25.7 mg/kg), chlorcyclizine HCl (37.3 mg/kg), Na hexabarbital (25.8 mg/kg), or antipyrine (18.8 mg/kg). The 3, 4-benzpyrene was administered at a dose of 100 μg/kg. All agents were given in water except 3, 4-benzpyrene which was given in corn oil. Control groups were similarly injected with water or corn oil.

Twenty-four hr after the last injection of the pretreatment agent the mice were sacrificed by decapitation and bled. Livers were excised, placed in ice-cold 0.2 *M* potassium phosphate buffer (pH 7.4), blotted on filter paper and homogenized in 2 vol of either this same buffer or in 0.1 m*M* EDTA in 0.25 *M* sucrose solution. The homogenates were centrifuged at 9000*g* for 20 min in a Sorvall refrigerated centrifuge. Supernates were readjusted to their original volumes with the corresponding media, and recentrifuged for 60 min at 105,000*g* in a Spinco Model L refrigerated ultracentrifuge. The remaining pellets containing microsomes were resuspended to an approximate protein concentration of 1 mg/ml, either in the phosphate buffer or in a 0.2 *M* tris(hydroxymethyl)aminomethane (Tris) buffer (pH 7.4) for the EDTA prepared fraction. Microsomal protein was determined according to the method of Lowry *et al.* (5).

For the cytochrome P-450 determination, 3 ml of both sample and reference microsomal suspensions in the Tris buffer were reduced with sodium dithionite. The sample was bubbled with carbon monoxide for 30 sec, and absorption at 450 mμ was recorded in the Aminco Chance Dual Wavelength recording spectrophotometer. Cytochrome P-450 concentration was calculated using the extinction coefficient of 91 mM^{-1} cm^{-1} (6). Either aminopyrine or aniline to a final concentra-

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TABLE I. The Influence of Inducing Agents on Microsomal Protein per Gram of Liver (A) and per Liver (B).[a]

	Adult				Weanling			
	Fed; (%)		Starved; (%)		27% Casein; (%)		8% Casein; (%)	
Pretreatment	A	B	A	B	A	B	A	B
Phenobarbital	+30	+60	+22[c]	+40[c]	+23	+70	+2[c]	+48
Chlorcyclizine	+48	+110	+43[c]	+74	+24	+85	+27[c]	+81
Hexobarbital	+12[c]	+44[c]	−5[c]	—	+2[c]	+21	−8[c]	+18[c]
Antipyrine	+13	+51	−2[c]	+3[c]	+12[c]	+46	+8[c]	+36
3,4-Benzpyrene	−8[c]	+3[c]	+18	+30[c]	−16[c]	−5[c]	+12	+15[c]
	Controls (mg of microsomal protein/g of liver)							
	Fed		Starved		27% Casein		8% Casein	
Water	5.11 ± 0.07[b]		4.61 ± 0.10		4.64 ± 0.06		3.84 ± 0.12	
Corn oil	5.26 ± 0.25		4.38 ± 0.08		4.86 ± 0.16		3.69 ± 0.01	
	Controls (mg of microsomal protein/liver)							
Water	11.01 ± 0.09[b]		5.25 ± 0.20		8.24 ± 0.36		3.84 ± 0.17	
Corn oil	11.21 ± 0.32		4.64 ± 0.16		8.31 ± 0.23		3.80 ± 0.02	

[a] Values indicate percentage difference between control mean, based on 4 separate determinations from livers of 15 to 20 mice, and experimental mean, based on 2 separate determinations from livers of 30 to 40 mice.

[b] Mean ± standard deviation of the mean.

[c] $p(t) =$ NS; all other values $p(t) \leq 0.05$.

tion of 3.8 m*M* was added to 3 ml of the microsomes suspended in the 0.2 *M* Tris buffer and the difference spectra was recorded for the determination of substrate binding to cytochrome P-450.

Results. The microsomal protein content per gram of liver and in the total liver was decreased by starvation and by feeding the 8% casein diet (Table I). Chlorcyclizine, a potent enzyme inducer, caused a significant increase in microscomal protein in the fed adult mice and in the weanlings fed the 27% casein diet. It was less effective in the starved adults and in the weanlings fed the low protein diet. Phenobarbital, an equally effective microsomal enzyme inducer, acted similarly. Hexobarbital only increased the total microsomal protein in the 27% casein-fed weanlings, whereas antipyrine increased it in all but the starved adult mice. The administration of 3, 4-benzpyrene resulted in an increase in microsomal protein per gram of liver in both the starved adult mice and in the weanlings fed the low protein diet.

Cytochrome P-450 levels per gram and per liver were decreased by starvation as well as by feeding the low protein diet (Table II). Phenobarbital and chlorcyclizine both caused marked increases in the concentration of this cytochrome in all groups studied. The largest percentage increases were found in the weanling animals regardless of their dietary intake. Hexobarbital increased the cytochrome P-450 level per gram of liver in all groups while antipyrine did so in all but the weanling mice fed the 8% casein diet. The 3, 4-benzpyrene was primarily active in the weanling mice fed the 27% casein diet.

When the amount of cytochrome was calculated per milligram of microsomal protein, it was again evident that chlorcyclizine was the most effective inducer and that its influence was very pronounced in the starved and in the weanling groups. With phenobarbital pretreatment, the largest increases were also noted in the weanling animals.

TABLE II. The Influence of Inducing Agents on Cytochrome P-450 per Gram of Liver (A), per Liver (B), and per mg of Microsomal Protein (C).[a]

	Adult						Weanling					
	Fed; (%)			Starved; (%)			27% Casein; (%)			8% Casein; (%)		
	A	B	C	A	B	C	A	B	C	A	B	C
Phenobarbital	+125	+175	+70	+150	+190	+105	+195	+285	+130	+180	+300	+150
Chlorcyclizine	+130	+265	+70	+225	+310	+130	+230	+360	+145	+220	+340	+135
Hexobarbital	+44	+90	+30	+60	+75	+75	+44	+60	+35	+42	+78	+48
Antipyrine	+45	+93	+26	+35	+44	+40	+37	+65	+17[c]	+12	+32[c]	—
3,4-Benzpyrene	+12[c]	+15[c]	+27	—	+10[c]	+14[c]	+31	+32	+52	+18[c]	+30[c]	+12[c]

	Controls (mμM cytochrome P-450/g liver)			
	Fed	Starved	27% Casein	8% Casein
Water	4.90 ± 0.06[b]	4.33 ± 0.14	3.21 ± 0.07	2.80 ± 0.05
Corn oil	4.90 ± 0.32	4.40 ± 0.17	3.01 ± 0.11	2.70 ± 0.08
	Controls (mμM cytochrome P-450/liver)			
Water	10.56 ± 0.14[b]	4.92 ± 0.19	5.72 ± 0.35	2.80 ± 0.11
Corn oil	10.93 ± 0.51	4.66 ± 0.26	5.15 ± 0.22	2.78 ± 0.10

[a] Values indicate percent differences between control mean, based on 4 separate determinations from livers of 15 to 20 mice, and experimental mean, based on 2 separate determinations from livers of 30 to 40 mice.

[b] Mean ± standard deviation of the mean.

[c] $p(t) = \text{NS}$; all other values $p(t) \leq 0.05$.

TABLE III. Spectral Binding Patterns of Aniline to Cytochrome P-450.

Pretreatment	Adult; (nm)				Weanling; (nm)			
	Fed		Starved		27% Casein		8% Casein	
	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum	Maximum	Minimum
Water	429	396	427	383[b]	428	395	425	391
Phenobarbital	428	393	430	394	433	401	427	391
Chlorcyclizine	429	396	430	394	432	399	433	397
Hexobarbital	428	397	428	396	430	392	431	395
Antipyrine	428	397	429	395	430	394	428	393
Corn oil	428	394	428	384[b]	429	397	426	391
3,4-Benzpyrene	426[a]	391	423[a]	392	431	396	429	393

[a] Outlier from max (428–433); USP XVII; 844 (1965).
[b] Outlier from min (391–401); USP XVII; 844 (1965).

A binding pattern of aminopyrine to cytochrome P-450 could only be obtained in the starved adult mice pretreated with either chlorcyclizine or phenobarbital. Binding patterns of aniline to cytochrome P-450 were recorded in all control and pretreatment groups (Table III). The aniline difference spectra were reproducable (wavelength difference constant peak to trough) when the entire study was repeated a second time. Analysis for outliers indicated that the only differences in binding patterns are in the water and corn oil controls of the starved adult mice and in the two adult groups pretreated with 3, 4-benzpyrene. In the weanling groups, 3, 4-benzpyrene administration did not alter the aniline difference spectra from that seen with other pretreatments.

Discussion. In the earlier study the starved adult and weanling mice had much longer hexobarbital sleeping times than the chow-fed adult mice. Consistent with this was the current observation that the livers from the starved adults and weanling mice were smaller and contained less microsomal protein and cytochrome P-450 than the chow-fed adult mice.

It was evident from this study that although the restriction of the diets did influence the ability of agents to stimulate microsomal protein synthesis, it had less effect on the synthesis of cytochrome P-450. With the more potent stimulators, there was an increased synthesis of the cytochrome in the starved adults. Approximately the same percentage increases were found in the low and high protein-fed weanling mice pretreated with phenobarbital or chlorcyclizine. These agents were the least active in the chow-fed adult mice. With the weaker agents, however, dietary restrictions tended to decrease the synthesis of the cytochrome.

On an equimolar basis, chlorocylizine was the most effective of the agents studied in increasing the cytochrome P-450 level per milligram of microsomal protein. When hexobarbital sleeping time was studied under the same experimental conditions, chlorcyclizine was also the most effective in shortening sleeping time. Although antipyrine administration did not cause a significant change in the cytochrome P-450 level per

milligram of microsomal protein in the weanling mice, it did significantly decrease hexobarbital sleeping time. Hence, it is obvious that there are other factors besides the cytochrome level which ultimately determine the rate of hexobarbital metabolism *in vivo*.

With 3, 4-benzpyrene, there was also no correlation between sleeping time observed and the cytochrome P-450 per milligram of microsomal protein. This agent is known to increase the detoxication of zoxazolamine but not to affect the metabolism of hexobarbital. In the adult fed mice, the cytochrome concentration significantly increased but 3, 4-benzpyrene had no effect on hexobarbital sleeping time in these adult mice. In weanling mice fed 27% casein, however, 3, 4-benzpyrene increased the cytochrome concentration and it decreased hexobarbital sleeping time in these mice 75%. This may indicate that 3, 4-benzpyrene induces the synthesis of a different type of cytochrome in adult and weanling mice. Polycyclic hydrocarbons like 3, 4-benzpyrene are believed to induce the synthesis of a different form of cytochrome P-450 (cytochrome P_1 450) in animals (8).

The aniline binding difference spectra recorded from microsomes obtained from weanling mice pretreated with 3, 4-benzpyrene was no different from that obtained with other pretreatments. However, in the adult mice, the aniline spectra in the 3, 4-benzpyrene group were slightly different from those seen with other agents. A similar peak shift towards shorter wavelengths in the ethylisocyanide difference spectra has been noted in adult mice pretreated with 3-methylcholanthrene (9). This difference in the binding spectra may be related to the induction of primarily P_1 450 in adult mice by these agents. If little or no P_1 450 is produced in weanling mice, further studies with such animals may be of value in elucidating the difference between the nature of this cytochrome and that of cytochrome P-450.

Summary. Pretreating mice for 3 days with equimolar doses of various inducing agents resulted in an increase in liver size, microsomal protein, and cytochrome P-450. The largest increases were produced by chlorcyclizine and phenobarbital and could be correlated with a decrease in hexobarbital sleeping time noted in a previous study. These agents were more active in weanling and dietary restricted mice than in fed adult mice. Hexobarbital and antipyrine caused smaller increases in microsomal protein and cytochrome P-450 and were not as effective in decreasing hexobarbital sleeping time. Pretreatment with 3, 4-benzpyrene increased the cytochrome concentration in both the fed adult and 27% casein-fed weanling mice but decreased hexobarbital sleeping time only in the weanling mice. The microsomes derived from adult mice pretreated with 3, 4-benzpyrene gave an aniline difference binding spectrum which differed from that seen in the weanling mice.

1. Conney, A. H., Pharmacol. Rev. **19**, 317 (1967).

2. Lee, N. H., Hospador, M. A., and Manthei, R. W., Proc. Soc. Exp. Biol. Med. **125**, 153 (1967).

3. Weatherholtz, W. M., Campbell, T. C., and Webb, R. E., J. Nutr. **98**, 90 (1969).

4. Hospador, M. A., and Manthei, R. W., Proc. Soc. Exp. Biol. Med. **128**, 130 (1968).

5. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. **193**, 265 (1951).

6. Omura, T., and Sato, R., J. Biol. Chem. **239**, 2379 (1964).

7. Conney, A. H., Davison, C., Gastel, R., and Burns, J. J., J. Pharmacol. Exp. Ther. **130**, 1 (1960).

8. Parli, C. J., and Mannering, G. J., Mol. Pharmacol. **6**, 178 (1970).

9. Alvares, A. P., Schilling, G., and Levin, W., J. Pharmacol. Exp. Ther. **175**, 4 (1970).

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