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GLUTATHIONE IN THE DEVELOPING MOUSE LIVER—I.

DEVELOPMENTAL CURVE AND DEPLETION AFTER ACETAMINOPHEN TREATMENT*

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Abstract—The developmental aspects of glutathione in the mouse fetal liver and the relative roles of glutathione in maternal liver, placenta and fetal liver in the protection against possible harmful effects of acetaminophen were studied. The glutathione concentration in the fetal mouse liver is significantly less than that in adult liver and reaches the adult levels about 10 days after birth. The rate of depletion of glutathione after acetaminophen administration reaches the adult levels about 15 days after birth. Treatment with 3-methylcholanthrene (MC) enhances the rate of glutathione depletion in both maternal and fetal livers of the genetically responsive C57BL/6N mouse, but not in the gentically nonresponsive AKR/N mouse. No teratogenic effects of acetaminophen were observed at doses between 100 and 250 mg kg^{-1} given between days 6 and 13 of gestation.

Although glutathione (γ-glutamyl-L-cysteinylglycine) is invariably found in nearly all living cells, its biological function is not fully understood [1]. It is thought to be essential for the protection of thiol groups in proteins against the toxic effects of foreign compounds, including metals and hydrogen peroxide, through the action of glutathione peroxidase and transferases [1, 2]. Glutathione is also involved in several enzyme reactions as a substrate or as a coenzyme [1].

The utilization of glutathione for the biosynthesis of glutathione derivatives of toxic foreign compounds, which are less toxic than the parent compound, is of obvious benefit to the organism. This protective role of glutathione has recently been emphasized by work done on the mechanism of acetaminophen (*p*-hydroxyacetanilide, Tylenol and Paracetamol) -induced hepatotoxicity in experimental animals and on the protective effect of cysteamine treatment in acetaminophen overdoses in man [3, 4]. Acetaminophen is a widely used analgesic particularly in pediatric medicine, but little information is available on the role of glutathione in protecting the fetus and the neonate against possible harmful effects of drugs such as acetaminophen. The purpose of the present investigation was to study the developmental aspects of glutathione in the mouse fetal liver and the relative roles of glutathione in maternal liver, placenta and fetal liver in the protection against possible harmful effects of acetaminophen.

*A portion of this work was presented at the Federation of American Societies for Experimental Biology, Atlantic City, April 1975 [G. H. Lambert and S. S. Thorgeirsson, *Fedn Proc.* **34**, 774 (1975)].

†Abbreviations used are: B6, the inbred C57BL/6N mouse strain; AK, the inbred AKR/N mouse strain; MC, 3-methylcholanthrene.

MATERIAL AND METHODS

Generally labeled [^{3}H]acetaminophen (sp. act. 270 mCi/m-mole) was obtained from New England Nuclear Corp. Grade A glutathione, reduced pyridine nucleotides (NADPH and NADH), and grade B *N*-ethylmaleimide were obtained from CalBiochem. Yeast type III highly purified glutathione reductase and 5,5'-dithiobis-(2-nitrobenzoic acid) were purchased from Sigma. Acetaminophen (*p*-hydroxyacetanilide) was obtained from Eastman Kodak (Rochester, N.Y.). All other chemicals were of the highest available purity.

The inbred strains of mice, B6 and AK,† were obtained from the National Institutes of Health, Animal Production Section. Mice were allowed water and food (Purina Lab Chow) *ad lib.* One male and four females were allocated/cage, and vaginal plugs were checked for every day between 8:00 and 9:00 a.m. The day the plug was found was day "zero" of gestation. The plugged females were then placed in separate cages with a maximum of five females/cage. The environment of the animal room was controlled as rigidly as possible, as previously described [5].

MC was injected intraperitoneally in corn oil at a dose of 80 mg kg^{-1} body weight. Control animals received corn oil only. Animals were sacrificed 48 hr after the treatment. Oxidized and reduced glutathione were assayed on a Gilford 240 or Aminco DW-2 spectrophotometer according to the method by Tietze [6]. Covalent binding *in vivo* of acetaminophen to tissue proteins was estimated as described by Jollow *et al.* [7].

The teratogenicity of acetaminophen was studied by treating the pregnant mice on days 6–13 of gestation with acetaminophen, 100–250 mg/kg intraperitoneally. The pregnant mice were sacrificed on day 18: the fetuses were weighed, crown-rump measure-

Table 1. Reduced and oxidized glutathione contents in maternal liver, fetal liver, and placenta from the AK mouse*

	Reduced glutathione (μmoles/g tissue)	Oxidized glutathione (μmoles/g tissue)	% of total
Maternal liver	8.73 ± 1.36	0.44 ± 0.28	4.80
Fetal liver†	3.00 ± 0.16	0.08 ± 0.02	2.45
Placenta†	0.85 ± 0.13	0.05 ± 0.01	5.44

* Weighed tissue was homogenized in 5% trichloroacetic acid/0.1 N HCl. After centrifugation the supernatant solutions were extracted five times at 0° with equal volumes of ether and divided into two portions, one of which was used without further treatment for the determination of total glutathione. The second portion was incubated for 1 hr at 25° with an equal volume of 0.04 M *N*-ethylmaleimide in phosphate-EDTA buffer. After removal of the unreacted *N*-ethylmaleimide by eight extractions with ether, the solution was assayed for oxidized glutathione. Both reduced and oxidized glutathione were assayed according to the method of Tietze [6]. Values are recorded as means ± S. E. M. for six experiments.

† Day 18 of gestation.

ments were taken and the fetuses were processed as described by Monie *et al.* [8].

The distribution and rate of metabolism of acetaminophen in the pregnant mouse were determined by administering [^{3}H]acetaminophen and measuring the unchanged drug that remained in maternal blood and liver, placenta and fetal liver at various time intervals according to the method of Mitchell *et al.* [9].

RESULTS

The concentration of glutathione in the fetal liver and placenta at term was 33 and 10%, respectively, of the maternal liver (Table 1). The percentage of oxidized glutathione of the total glutathione content was similar in placenta, maternal and fetal livers, ranging from 2.45 to 5.44 per cent of the total glutathione content. These results are in agreement with the amount of oxidized glutathione found in adult rat kidney and liver [6].

The developmental curve of glutathione content in the liver for the AK and B6 mice is shown in Fig. 1. The glutathione developmental curve for the B6 mouse was not significantly different from that of the AK mouse. Glutathione concentration in the fetal liver at day 15 of gestation was about 12% of the adult level, and increased to about 33% at birth. After birth, the glutathione level rapidly increased to reach adult levels at about 10 days of age.

The distribution and disappearance of acetaminophen in maternal blood and liver, fetal liver and placenta are shown in Fig. 2. Similar concentrations and rates of disappearance were found in all tissues, indicating that mother, placenta and fetus represent a "single pharmacokinetic compartment" for acetaminophen.

Acetaminophen depletes glutathione in mouse liver after large doses [9]. Figure 3 shows the depletion of glutathione in maternal liver after administration of 250, 500 or 1000 mg kg^{-1} of acetaminophen intraperitoneally. Glutathione is depleted to about 15 per cent of control value by 2 hr after a 250 mg kg^{-1} dose of acetaminophen, but returned to 63 and 120 per cent of control values within 3 and 5 hr respectively. The glutathione level in the liver 24 hr after the 250 mg kg^{-1} dose was 165 per cent of control values. Both 500 and 1000 mg kg^{-1} of acetaminophen

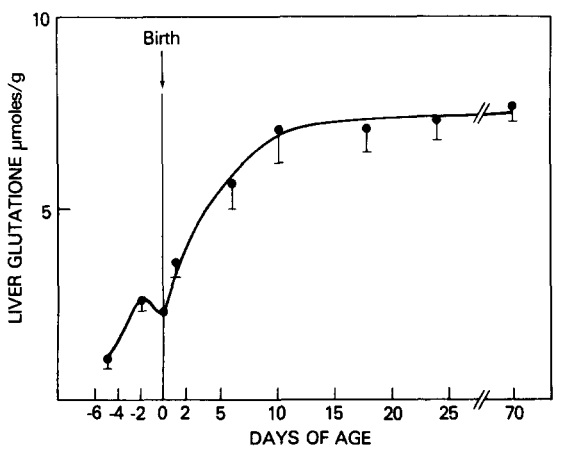

Fig. 1. Developmental curve of glutathione content in the livers from AKR/N and C57BL/6N mice. The points on this and subsequent figures are means ± S. E. M.

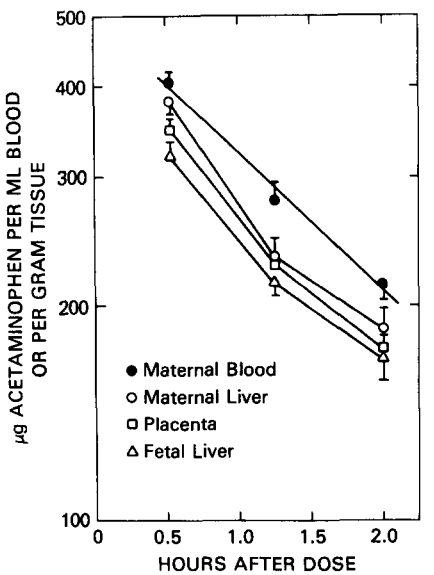

Fig. 2. Disappearance of acetaminophen (single dose 500 mg kg^{-1} i.p.) from maternal blood and liver, fetal liver and placenta in 18-day-pregnant AKR/N mouse.

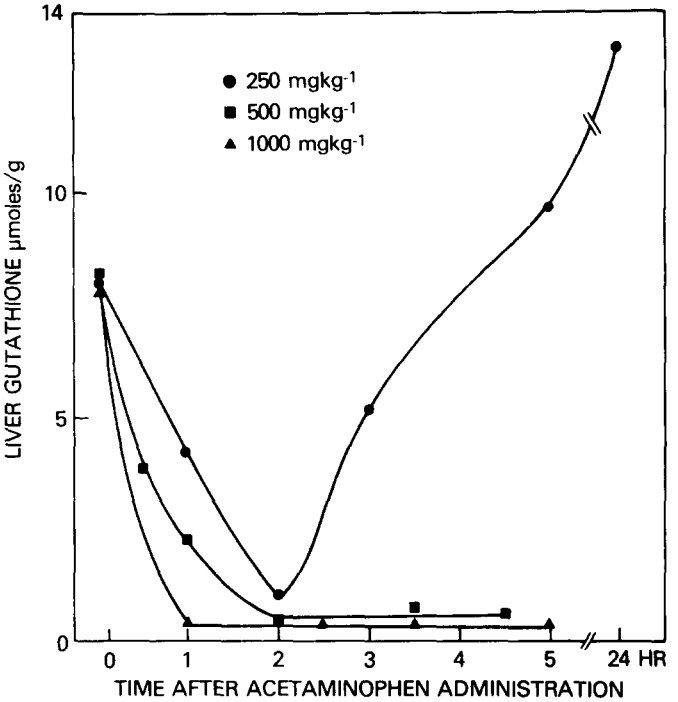

Fig. 3. Depletion of glutathione in maternal liver after administration of 25, 500 or 1000 mg kg^{-1} of acetaminophen i.p. to 18-day-pregnant AKR/N mouse.

depleted glutathione in the liver to less than 10 per cent of control values by 2 hr after administration, and the glutathione level remained at this level for the next 3 hr; in most instances, these mothers died within 12 hr. This depletion is in contrast to the rebound in glutathione content observed after the 250 mg kg^{-1} dose of acetaminophen, indicating possible impairment of glutathione synthesis presumably caused by the liver necrosis seen after 500 and 1000 mg kg^{-1} doses [5, 9].

Glutathione levels in fetal livers at 18 days of gestational age after administration of 250, 500 or 1000 mg kg^{-1} of acetaminophen to the mother are shown in Fig. 4. The fetuses were removed from the mothers whose liver glutathione depletion was shown in Fig. 3. In contrast to the glutathione depletion found in maternal liver, no significant depletion of glutathione in fetal liver was detectable after the 250 mg kg^{-1} dose of acetaminophen. The glutathione level was decreased to about 90 and 50 per cent of control value by 5 hr after 500 and 1000 mg kg^{-1} of acetaminophen respectively.

Figure 5 shows the depletion of liver glutathione in age-matched newborn and adult mice after acetaminophen administration. Acetaminophen (500 mg kg^{-1}) depleted glutathione by 0–15 per cent 0.5 hr after administration during the first 10 days of life. The rate of glutathione depletion was thereafter rapidly increased with age, and reached adult values by approximately day 15. It is of interest, in terms of development, to note that glutathione content in the liver reached adult levels at about day 10 (Fig.

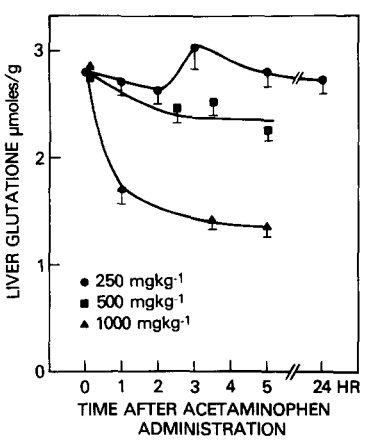

Fig. 4. Glutathione levels in fetal livers from AKR/N mouse at 18 days of gestational age after administration of 250, 500 or 1000 mg kg^{-1} i.p. to the mother.

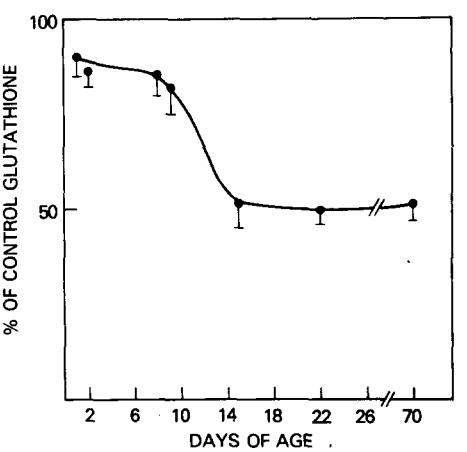

Fig. 5. Developmental curve for the depletion of liver glutathione in the AKR/N mice 0.5 hr after acetaminophen administration (single dose 500 mg kg^{-1} i.p.).

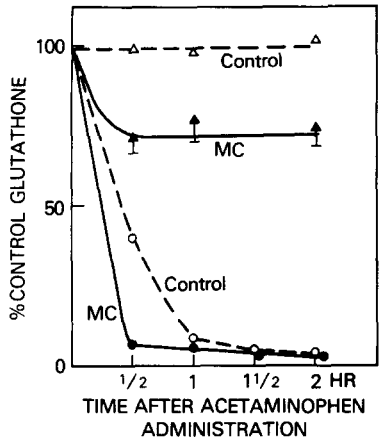

Fig. 6. Effect of MC treatment during pregnancy on glutathione depletion in maternal [(O--O) control and (●——●) MC-treated] and fetal [(△--△) control and (▲——▲) MC-treated] livers from C57BL/6N mice at day 18 of gestation after administration of acetaminophen (500 mg kg^{-1} i.p.).

1), whereas the rate of glutathione depletion reaches adult levels by about day 15 of life.

Polycyclic hydrocarbons such as MC have been shown to induce the cytochrome P_1-450-mediated monooxygenase system(s) in liver and other tissues of certain inbred strains of mice but not in others [10]. Aryl hydroxylase, 2-acetylamino-fluorene *N*-hydroxylase and liver toxicity of acetaminophen have previously been reported to be induced by MC treatment of the "responsive" B6 mouse [5]. No increase in either enzyme activity or acetaminophen toxicity is found after MC treatment of the nonresponsive strains such as AK or DBA/2N [5, 10]. The effect of MC treatment during pregnancy on glutathione depletion in B6 maternal and fetal livers at day 18 of gestation after the intraperitoneal administration of acetaminophen (500 mg kg^{-1}) is shown in Fig. 6. There was a significant increase in the rate of glutathione depletion in the B6 maternal liver after MC, and the fetal liver was depleted at 0.5 hr to about 70 per cent of control value. No depletion of glutathione was seen in the fetal livers from control mice not receiving MC.

Acetaminophen is metabolized both *in vivo* and *in vitro* by a cytochrome P-450-dependent monooxygenase(s) into a reactive, and presumably toxic, arylating metabolite [7, 11]. Furthermore, glutathione protects against acetaminophen-induced hepatotoxicity by reacting with the toxic metabolite and thereby preventing it from covalently binding to tissue macromolecules. Covalent binding *in vivo* of acetaminophen metabolite(s) to cellular macromolecules in the placenta, maternal liver and fetal liver from B6 and AK mice is shown in Table 2. MC treatment of the responsive B6 mouse increased the covalent binding in maternal liver 2-fold, whereas the binding to fetal liver was significantly decreased. Binding to placenta is not affected by MC treatment. No significant change in covalent binding was found after MC treatment of the nonresponsive AK mouse, and both placenta and fetal liver were similarly unaffected.

We found no teratogenic effects of acetaminophen at doses between 100 and 250 mg kg^{-1} given between days 6 and 13 of gestation. There were no significant differences in the number of fetal resorptions, in fetal weight or in fetal size of acetaminophen-treated mice compared with control mice. No bony or soft tissue malformations were observed in ten litters.

DISCUSSION

In this paper we have shown that the glutathione concentration in the fetal mouse liver is significantly less than that in adult liver and reaches adult levels about 10 days after birth (Fig. 1). The rate of depletion of glutathione after acetaminophen administration reaches adult levels about 15 days after birth (Fig. 5). MC treatment enhances the rate of glutathione depletion in both maternal and fetal livers of the genetically responsive B6 mouse, but not in the genetically nonresponsive AK mouse. In addition, covalent binding *in vivo* of acetaminophen metabolite(s) in maternal B6 liver is increased after MC treatment, but binding in fetal liver is decreased. No changes in the covalent binding *in vivo* of acetaminophen were found for the AK mouse (Table 2).

Several studies have indicated that one of the more important roles of endogenous glutathione may be to protect vital nucleophilic sites in the hepatocytes

Table 2. Effect of MC treatment on covalent binding *in vivo* of acetaminophen to mouse tissue protein*

Strain	Previous treatment	Dose of acetaminophen (mg kg^{-1})	Covalently bound acetaminophen (pmoles/mg tissue)		
			Maternal liver	Fetal liver	Placenta
B6	None	500	1100 ± 106	230 ± 15	130 ± 20
	MC	500	2250 ± 140†	140 ± 25†	130 ± 17
AKR	None	500	660 ± 73	83 ± 15	93 ± 20
	MC	500	560 ± 65	73 ± 18	86 ± 23
	None	1000	1200 ± 135	260 ± 25	280 ± 80
	MC	1000	1400 ± 210	220 ± 35	210 ± 46

* Mice at day 18 of gestation were killed 2 hr after injection of acetaminophen ([^{3}H]acetaminophen generally labeled; sp. act. 270 mCi/m-mole) and samples were taken from the livers, placenta and psoas muscles. The tissues were homogenized in 0.9% NaCl potassium phosphate buffer, pH 7.4, and aliquots were added to 2 ml of 1.0 M trichloroacetic acid. The subsequent extractions of the proteins with trichloroacetic acid and methanol were performed as described by Jollow *et al.* [7]. The values shown are corrected by subtracting the presumably non-specifically bound radioactivity found in the samples from psoas muscle. Data are recorded as means ± S. E. M. of four experiments.

† $P < 0.05$.

and other tissues from electrophilic attacks by alkylating and arylating metabolites of drugs and other foreign compounds [1, 7, 9]. It therefore would be of obvious benefit to the developing fetus and the neonate if the synthetic pathways of glutathione and the glutathione transferases appeared earlier in development than the monooxygenases—which are known, in some instances, to metabolize drugs and foreign compounds in to reactive alkylating and arylating intermediates [10–12]. The present study indicates that this developmental sequence may indeed be the case. The adult level of the hepatic glutathione, which represents primarily the development of the synthetic pathways, is reached about 10 days after birth (Fig. 1), whereas the adult rate of glutathione depletion after a large dose of acetaminophen occurs about 15 days after birth (Fig. 5). Acetaminophen, and several other *N*-acetylarylamines, are metabolized by cytochrome P-450-dependent monooxygenase(s) into a reactive arylating metabolite capable of depleting glutathione and covalently binding to liver proteins [3, 7]. Neither depletion of glutathione nor covalent binding to cellular macromolecules occurs if cytochrome P-450-dependent metabolism of acetaminophen is blocked [7, 13]. Therefore, the rate of glutathione depletion after acetaminophen administration reflects primarily the development of the monooxygenase(s) capable of metabolizing acetaminophen into a reactive electrophile.

This concept that the glutathione system appears earlier in development than the monooxygenase system is further supported by our results with covalent binding of acetaminophen. Covalent binding *in vivo* of acetaminophen in B6 maternal liver is increased after MC treatment, but in contrast the covalent binding in the fetal liver is decreased. The monooxygenase(s) in the fetal liver responsible for acetaminophen metabolism is clearly induced by MC treatment of the mother as evidenced by the decrease in the fetal liver glutathione (Fig. 5). Hence, the decrease in the covalently bound acetaminophen to fetal liver proteins seems to indicate that conjugation of glutathione and the electrophilic metabolite of acetaminophen, presumably catalyzed by a transferase, is more effective after MC treatment. Induction by MC of glutathione transferases has been observed in the adult B6 mouse,* and our results indicate that MC treatment in the fetus and early neonate may induce the glutathione transferases to a greater extent than the cytochrome P-450-dependent monooxygenases in the fetus.

The importance of glutathione conjugation in protecting the liver against the arylating metabolite of acetaminophen in the neonate and the young child is at present unknown. Conjugation of drugs, including acetaminophen and its analog phenacetin, with glutathione occurs in the adult man, but enzyme activities are low compared with those in other species [14, 15]. Thus, any extrapolation of the present study to the clinical areas of pediatric pharmacology and toxicology must await an evaluation of the importance of glutathione conjugative reactions in both normal children and in children with various illnesses.

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*J. S. Felton and D. W. Nebert, personal communication.

REFERENCES

1. E. Boyland and L. F. Chasseaud, *Adv. Enzymol.* **32,** 173 (1969).
2. G. C. Mills, *Archs Biochem. Biophys.* **86,** 1 (1960).
3. J. R. Mitchell, D. J. Jollow, W. Z. Potter, J. R. Gillette and B. B. Brodie, *J. Pharm. exp. Ther.* **187,** 211 (1973).
4. L. F. Prescott, R. W. Newton, C. P. Swainson, N. Wright, A. R. W. Forrester and H. Matthew, *Lancet* **1,** 588 (1974).
5. S. S. Thorgeirsson, J. S. Felton and D. W. Nebert, *Molec. Pharmac.* **11,** 159 (1975).
6. F. Tietze, *Analyt. Biochem.* **27,** 502 (1969).
7. D. J. Jollow, J. R. Mitchell, W. Z. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187,** 197 (1973).
8. I. W. Monie, K. G. Kho and J. Morgan, *Supplement to Teratology Workshop Manual*, p. 163. University of Chicago Press, Berkeley, California (1965).
9. J. R. Mitchell, D. J. Jollow, W. F. Potter, D. C. Davis, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187,** 185 (1973).
10. D. W. Nebert, J. R. Robinson, A. Niwa, K. Kumaki and A. P. Poland, *J. Cell Physiol.* **85,** 393 (1975).
11. D. M. Jerina and J. W. Daly, *Science, N.Y.* **185,** 573 (1974).
12. P. Sims and P. L. Grover, *Adv. Cancer Res.* **20,** 166 (1974).
13. W. Z. Potter, D. C. Davis, J. R. Mitchell, D. J. Jollow, J. R. Gillette and B. B. Brodie, *J. Pharmac. exp. Ther.* **187,** 203 (1973).
14. J. R. Mitchell, S. S. Thorgeirsson, W. Z. Potter, D. J. Jollow and H. Keiser, *Clin. Pharmac. Ther.* **16,** 676 (1974).
15. D. J. Jollow, S. S. Thorgeirsson, W. Z. Potter and J. R. Mitchell, *Pharmacology* **12,** 251 (1974).