INTRODUCTION

GSH$^\alpha$ (L-$\gamma$-glutamyl-L-cysteinylglycine) is widely distributed in animal and plant cells. It is found primarily intracellularly in relatively high concentrations ranging from 0.5 to 10.0 mM and plays an integral role in a large number of biological functions (8). Some of these include: (a) protection of cell membranes by the maintenance of essential thiol (SH) groups of proteins and other molecules; (b) destruction of hydrogen peroxide and other peroxides or of radiation-induced free radicals; (c) translocation of amino acids, small peptides, and amines across cell membranes; and (d) detoxification of xenobiotics via the conjugation of the "reactive" metabolite(s) with GSH and subsequent elimination of the conjugate as the mercapturic acid.

The intracellular synthesis of GSH and its degradation are linked by a series of 6 enzyme-catalyzed reactions, collectively known as the $\gamma$-glutamyl cycle (8). The synthesis of GSH takes place in 2 successive steps catalyzed by the soluble enzymes, $\gamma$-glutamylcysteine synthetase and GSH synthetase, respectively, while the major pathway of degradation is catalyzed by the membrane-bound enzyme GGTP. Although the synthesis and degradation of GSH have been studied extensively in normal tissues (6, 8, 9, 11, 14, 17), regulation of GSH levels in the transformed cell or neoplastic tissue is poorly understood.

Normal hepatic tissues generally contain only small amounts of GGTP activity (1, 4, 17), but in the fetal and neonatal liver GGTP activity is considerably higher (4, 5, 15). The administration of various hepatocarcinogens results in marked increases in GGTP activity (up to 100-fold) in both preneoplastic and neoplastic livers (4, 5, 15). However, no studies concerning the activities of the 2 enzymes involved in the biosynthesis of GSH in these tissues, namely $\gamma$-glutamylcysteine synthetase and GSH synthetase, have appeared in the literature to date. In this paper we report the results of our studies concerning the regulation of the synthesis and degradation of GSH in the normal adult rat liver, in the fetal rat liver, and in the rapidly growing nondifferentiated transplantable Novikoff hepatoma.

MATERIALS AND METHODS

Diethyl maleate was purchased from Aldrich Chemical Co. (Milwaukee, Wis.). GSH reductase (yeast type III), L-$\gamma$-
glutamyl-p-nitroaniline, glycglycine, 3-carboxy-4-nitrophenyl disulfide, GSH, phosphoenolpyruvate (triclohexylammonium salt), amino acids, pyruvate kinase, ATP, and diphenylamine were obtained from Sigma Chemical Co. (St. Louis, Mo.). NAPDH was purchased from Boehringer Mannheim (Indianapolis, Ind.). [U-14C]Glutamate (225 mCi/mm) and [2-3H]glycine (5 to 15 Ci/mm) were obtained from New England Nuclear (Boston, Mass.). All other chemicals were the purest available commercial reagents. Taconic Farm Inc. (Germantown, N. Y.) provided us with pregnant Sprague-Dawley rats while male Sprague-Dawley rats (100 to 150 g) were obtained from the National Institutes of Health Animal Production Section and were fed water and basic Ralston Purina rat chow ad libitum. Pregnant rats were obtained 7 days prior to delivery, and fetal livers were taken from pregnant rats 3 days before delivery. Transplantable Novikoff hepatoma was obtained from Arthur D. Little, Inc. (Cambridge, Mass.) and maintained by either i.m. or i.p. transplantation in male Sprague-Dawley rats.

Glutathione Depletion. GSH depletion was achieved by administration of diethyl maleate (1000 mg/kg i.p.) to pregnant female rats (18-day term), rats bearing i.m.-transplanted Novikoff hepatomas on the hind legs, and control rats (2). The rats were sacrificed after various time intervals by decapitation, and GSH content was measured in fetal and adult liver and in the hepatoma.

Tissue Preparation. All tissues were removed between 8:30 a.m. and 10:30 a.m. except during the time-course experiments when animals were killed and tissues were removed at various times. Following exsanguination of the rats, samples of fetal liver, maternal liver, and livers from control and tumor-bearing animals were quickly removed, rapidly frozen in a hexane/dry ice bath, weighed, and immediately homogenized in 4 ml of 5% trichloroacetic acid/0.01 N HCl with a Willems Polytron (Brinkmann Instruments, Inc., Westbury, N. Y.). In animals bearing the transplanted hepatomas, tumors were carefully dissected from necrotic and hemorrhagic tissues and treated in a similar manner. The homogenates were centrifuged at 2000 × g for 10 min and total GSH content (oxidized and reduced) of the supernatant determined on a Beckman Model 25 spectrophotometer according to the procedure of Tietze (18).

DNA content was determined with the procedure of Schneider (13). Protein concentration was determined by the method of Lowry et al. (7) with crystalline bovine serum albumin as the protein standard.

Assay of Enzyme Activities. GGTP was assayed at 37° according to the method described by Orlowski and Meister (10), slightly modified, with γ-glutamyl-p-nitroaniline as the substrate and glycylglycine as the acceptor. The amount of γ-p-nitroaniline formed from substrate was determined colorimetrically at 410 nm. Since GGTP is a membrane-bound enzyme, it was necessary to use the microsomal fraction of both livers and hepatomas as the source of enzyme. Tissue samples were carefully removed as described above, minced, washed as free of hemoglobin as possible with ice-cold 300 mM sucrose/10 mM Tris/MgCl₂ buffer (pH 7.25), and homogenized at 0° in the same buffer (5/1, w/v) with a Willems Polytron. All subsequent steps were performed at 0–4°. The homogenates were centrifuged at 9000 × g for 20 min, and the resulting supernatant was carefully decanted and recentrifuged at 105,000 × g for 60 min in a Beckman Model L5-50 ultracentrifuge. The microsomal pellet was used to assay for transpeptidase activity, while the postmicrosomal supernatant fraction was used to assay for γ-glutamylcysteine synthetase and GSH synthetase activities.

GGTP synthetase and γ-glutamylcysteine synthetase activities were assayed by the methods of Davis et al. (3) with minor modifications. The standard assay for the radioisotopic synthesis of GSH from glutamate, cysteine, and glycine was performed in screw-cap scintillation vials at 37° for 60 min. The standard glutathione synthetase incubation mixture contained, in a total volume of 2 ml, the following reagents expressed in final concentrations: 10 mM [U-14C]glutamate (0.075 mCi/mm), 15 mM glycine, 10 mM cysteine, 4 mM phosphoenolpyruvate, 10 mM MgCl₂, 100 mM KCl, 100 mM Tris buffer (pH 7.8), 10 units of pyruvate kinase, and 4 mM ATP. In some experiments [2-3H]glycine (0.075 mCi/mm) was used instead of [14C]glutamate. The reaction was initiated by the addition of the soluble enzyme (2.5 mg/ml) and terminated by the addition of an 0.8-ml portion of the incubation mixture to a 3.2-ml solution of 12% trichloroacetic acid containing 25 mg of carrier GSH. The mixture was stirred vigorously, and the suspension was centrifuged to remove the precipitated protein. The procedure for the isolation of [14C]GSH from the supernatant was a slight modification of that used by Davis et al. (3). GSH was first precipitated as the cadmium salt and then as the cuprous mercaptide as reported by Davis et al. (3). The aqueous suspension of the cuprous mercaptide of GSH was centrifuged at 1000 × g for 5 min, and the supernatant was discarded. The precipitate was resuspended in 7 ml of deionized water and then centrifuged. This washing procedure was continued until no further radioactivity could be removed from the precipitate; usually 3 to 4 washes were sufficient. To each test tube were added 5 ml of scintillation cocktail (Aquasol; New England Nuclear), and each tube was vigorously stirred with a Vortex mixer. The Aquasol suspension of the GSH cuprous mercaptide was then transferred to 20-ml scintillation vials. Two successive 5-ml portions of Aquasol were then added to each test tube, the contents were thoroughly mixed, and they were added to the respective scintillation vials. Radioactivity was determined with a Packard Tri-Carb scintillation spectrophotometer. Counts were corrected for background and quench (external standardization) and converted to nmol of GSH on the basis of the specific activity of the starting [14C]glutamate or [2-3H]glycine.

The incubation conditions for the assay of γ-glutamylcysteine synthetase were essentially the same as those described for GSH synthetase except that glycine was omitted from the incubation mixture and [U-14C]glutamate was used. The same procedure that was used for the isolation of radiolabeled GSH was used for the isolation of γ-[14C]glutamylcysteine with GSH as the carrier for γ-glutamylcysteine. The amount of γ-[14C]glutamylcysteine formed was calculated on the basis of [14C]glutamate incorporated into product.

Enzyme activities of both γ-glutamylcysteine synthetase and GSH synthetase were expressed as nmol of radiolabel incorporated into product per mg soluble protein per 60 min at 37°.
RESULTS

Table 1 shows the total GSH content of various hepatic tissues as expressed per μg wet weight of tissue, per μg tissue protein, and per μg tissue DNA. GSH levels were similar in fetal liver and Novikoff hepatoma in all cases, and both were, whether expressed per wet weight, per tissue protein, or per tissue DNA, significantly lower (p < 0.05) than the GSH level in adult rat liver.

Following the administration of a single i.p. dose of diethyl maleate (1000 mg/kg), an agent known to deplete GSH levels rapidly in rat livers in vivo (2), GSH levels in the livers of adult control rats, and in transplanted Novikoff hepatomas were rapidly depleted (Chart 1). In the adult control rats hepatic GSH levels were depleted to less than 10% of control levels 30 min after diethyl maleate administration, remained maximally depleted for 3 to 4 hr after which they began to rise rapidly, returning to normal values after 6 hr and to 200% of normal values at 24 hr. In contrast to the control livers, GSH levels in the transplanted tumors were maximally depleted to 23% of control levels after 4 hr and showed a much slower rate of resynthesis reaching normal levels only after 24 hr. GSH levels in the hepatomas did not exceed normal levels after diethyl maleate administration. In data not shown hepatic GSH levels in rats with transplanted hepatomas followed a very similar time course for depletion and resynthesis of GSH following diethyl maleate as in the control rats.

Chart 2 shows a similar time course for the in vivo depletion and resynthesis of GSH in 18-day-old fetal rat liver following the i.p. administration of diethyl maleate to pregnant mothers. In the fetal livers GSH was rapidly depleted to 25% control levels after 3 hr and reached control values after 9 hr. GSH content in the fetal liver did not exceed normal values after diethyl maleate administration.

In agreement with the observations of others (4, 5, 15), the in vitro activity of GGTP in the liver tissues varied greatly (Table 2). In the Novikoff hepatoma GGTP activity was increased nearly 50-fold over that observed in the adult rat liver. Similarly, GGTP activity in the fetal rat liver was nearly 16 times as high as the transeptidase activity in the adult liver but only about 30 to 33% of the activity in the Novikoff hepatoma.

In addition to the differences in the activity of GGTP in the normal liver, fetal liver, and the Novikoff hepatoma, there were also marked differences in the in vivo activities of γ-glutamylcysteine synthetase and GSH synthetase in these tissues (Table 2). The incorporation of [14C]glutamate into γ-glutamylcysteine and GSH was taken as a measure of γ-glutamylcysteine synthetase and GSH synthetase, respectively. The formation of γ-[14C]glutamylcysteine from [14C]glutamate and cysteine occurred at a rate of 59.80 nmol per mg soluble protein per 60 min in the 100,000 × g supernatant from normal adult rat liver which was 3 to 4 times greater than the formation of γ-glutamylcysteine in either the fetal liver (20.6 ± 2.9 nmol/mg/60 min) or in the Novikoff hepatoma (14.0 ± 2.1 nmol/mg/60 min). The addition of 5 mm GSH to the γ-glutamylcysteine synthetase incubation mixtures resulted in significant inhibition (p < 0.05) of γ-glutamylcysteine formation in both the adult and fetal rat livers, and also in the Novikoff hepatoma.

The activity of GSH synthetase was 395.8 ± 31.9 nmol of GSH formed per mg soluble protein per 60 min in normal adult rat liver. This was nearly 3 times the rate for the formation of GSH in the fetal liver (133.8 ± 14.1 nmol/mg/60 min) and greater than 14 times the rate for the synthesis of GSH in the Novikoff hepatoma (27.6 ± 4.1 nmol/mg/60 min) (Table 2).

DISCUSSION

The present experimental results, which are in agreement with the observations of others (4, 5, 15), the in vitro activity of GGTP in the liver tissues varied greatly (Table 2). In the Novikoff hepatoma GGTP activity was increased nearly 50-fold over that observed in the adult rat liver. Similarly, GGTP activity in the fetal rat liver was nearly 16 times as high as the transeptidase activity in the adult liver but only about 30 to 33% of the activity in the Novikoff hepatoma.

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Table 2

In vitro activity of GGTP, \( \gamma \)-glutamylcysteine synthetase, and GSH synthetase in Novikoff hepatoma, adult liver, and fetal liver

The assays for GGTP, \( \gamma \)-glutamylcysteine synthetase, and GSH synthetase were performed as described in "Materials and Methods." Values for GGTP activity are expressed as nmol of p-nitroaniline liberated per mg microsomal protein per 30 min and represent the mean ± S.D. of 3 determinations on each of 3 animals. Values for \( \gamma \)-glutamylcysteine synthetase and GSH synthetase activity are expressed as nmol of radiolabel incorporated into product per mg soluble protein per 60 min, were corrected for extraction efficiency, and represent the mean ± S.D. of 5 determinations resulting from the use each time of the soluble protein fraction from the pooled homogenates of 4 adult animals (both control and Novikoff) and 30 fetuses.

<table>
<thead>
<tr>
<th>Tissue</th>
<th>GGTP (control)</th>
<th>GSH (5 mM)</th>
<th>GSH synthetase with glycine (10 mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adult liver</td>
<td>19.5 ± 2.0</td>
<td>59.8 ± 8.5</td>
<td>34.1 ± 3.3a</td>
</tr>
<tr>
<td>Fetal liver</td>
<td>306.0 ± 30b</td>
<td>20.6 ± 2.8</td>
<td>11.0 ± 1.0a</td>
</tr>
<tr>
<td>Novikoff hepatoma</td>
<td>906.0 ± 6.0b</td>
<td>14.0 ± 2.1</td>
<td>8.1 ± 1.6a</td>
</tr>
</tbody>
</table>

a Significantly different from control values (p < 0.05).
b Significantly different from the adult liver (p < 0.05).

with previous studies demonstrating the biochemical similarities between fetal and neoplastic tissue (16, 19), show that there are significant differences in the total GSH content as well as both in vivo and in vitro activities of the main enzymes involved in GSH biosynthesis and degradation between adult rat liver on the one hand and the Novikoff hepatoma and fetal rat liver on the other. The total GSH content in Novikoff hepatomas is 30% of that observed in adult rat liver (Table 1) and is in good agreement with the GSH content found in other rapidly growing hepatomas such as Yoshida and Morris 8994 (5). Similar GSH content is also found in fetal rat liver at 18 days of gestation (Table 1).

The in vivo time course for the depletion and resynthesis of GSH in the adult rat liver, in the fetal rat liver, and in the transplanted Novikoff hepatoma following the administration of diethyl maleate further illustrates the similarities between the fetal liver and Novikoff hepatoma, both of which differ from the adult liver (Charts 1 and 2). Diethyl maleate, an \( \alpha, \beta \)-unsaturated carbonyl compound, is known rapidly to conjugate GSH either enzymatically (via glutathione-S-alkene-transferase) or nonenzymatically in the liver and other organs to deplete GSH reversibly (2). Although the rate of GSH depletion is most rapid in the adult liver and also occurs to the greatest extent (90% depletion of GSH) in the normal adult liver than in either the fetal liver or in the transplanted hepatoma, this is probably due to differences in blood flow to the particular tissues; as a result, the fetal liver and the hepatoma do not receive as much diethyl maleate as does the adult liver.

The in vivo resynthesis of GSH is slower in both the fetal liver and the Novikoff hepatoma than in the adult rat liver. The characteristic overshoot in the restoration of GSH levels observed in the adult liver is absent from both the fetal liver and the hepatoma, indicating a difference in the control mechanism for GSH regulation in the adult liver versus fetal liver and hepatoma. Perhaps the high activity of GGTP found in both fetal liver and Novikoff hepatoma, in combination with the low activity of GSH synthetases, prevents the overshoot of GSH levels from occurring.

The adult rat liver possesses very low levels of GGTP activity, but, during the course of tumor formation following the administration of various hepatocarcinogens, transpeptidase activity in the primary hepatoma may reach levels up to 100 times normal liver values (5). We obtained similar results in that the in vitro GGTP activity in the Novikoff hepatoma was approximately 50 times the transpeptidase activity in the normal adult liver and 3 times the fetal activity.

The in vitro activity of the 2 synthetic enzymes in the biosynthesis of GSH are different in the normal liver, fetal liver, and the Novikoff hepatoma but appear to be under similar control (Table 2). \( \gamma \)-Glutamylcysteine synthetase activity in the normal rat liver is 3 to 4 times greater than the synthetase activity in either the fetal liver or the Novikoff hepatoma. Similarly, the synthesis of GSH is 14 times greater in the normal rat liver than it is in the Novikoff hepatoma and 3 times greater than it is in the fetal liver. The overall synthesis of GSH, however, reflects the activity of both \( \gamma \)-glutamylcysteine synthetase and GSH synthetase.

Regulation of GSH synthesis in vivo is probably under some type of feedback control since rat kidney \( \gamma \)-glutamylcysteine synthetase is markedly inhibited in vivo by GSH (12). The in vitro activity of \( \gamma \)-glutamylcysteine synthetase in the adult rat liver, in the fetal liver, and in the Novikoff hepatoma is inhibited to the same degree by 5 mM GSH (Table 2), suggesting that \( \gamma \)-glutamylcysteine synthetase has similar enzymatic characteristics in these tissues.

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Glutathione Synthesis and Degradation in Fetal and Adult Rat Liver and Novikoff Hepatoma

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