Regulation of Hepatic Glutathione Turnover in Rats In Vivo and Evidence for Kinetic Homogeneity of the Hepatic Glutathione Pool

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ABSTRACT The intracellular distribution of glutathione into kinetically distinct pools and the determinants of glutathione turnover were examined in vivo. Glutathione turnover was measured in individual, restrained rats with a biliary fistula by administration of acetaminophen to trap the previously labeled hepatic glutathione as an excretable acetaminophen adduct. Fasting for 48 h resulted in a decrease of hepatic glutathione from 4.7±0.9 to 3.6±0.8 μmol/g liver and a marked increase in the fractional rate of glutathione turnover from 0.19±0.04 to 0.43±0.07/h. Within 6 h following refeeding, the rate of glutathione turnover and the hepatic glutathione concentration returned to normal. The simultaneously determined specific activities of free intrahepatic glutathione and the acetaminophen-glutathione adduct in bile were identical, indicating that the hepatic glutathione pool is kinetically homogeneous. The synthesis of glutathione could, therefore, be estimated from the rate constant and the intrahepatic glutathione concentration. During fasting hepatic synthesis of glutathione increased from 0.86±0.17 to 1.50±0.23 μmol/g per h. In fed animals the administration of dibutyryl cyclic adenosine monophosphate and theophylline stimulated the rate of hepatic glutathione turnover similar to fasting. In contrast, glucose given intraduodenally to fasted animals decreased the rate of glutathione turnover. These data are consistent with the view that the increased glutathione turnover that occurs during fasting results from two mechanisms. Because of a decrease in the intrahepatic free glutathione/mixed disulfide ratio, which is apparently mediated by cyclic adenosine monophosphate, the free glutathione pool contracts and turns over more rapidly in order to maintain glutathione synthesis. In addition, glutathione consumption via the gamma-glutamyl cycle apparently is increased, which may be related to the increased uptake of amino acids for gluconeogenesis during fasting.

INTRODUCTION

More and more evidence accumulates proving that glutathione plays a fundamental role in cellular metabolism. As the most abundant intracellular sulfhydryl, glutathione is a key factor in the detoxification of electrophilic metabolites of xenobiotics (1, 2). As the cosubstrate for glutathione peroxidase, glutathione prevents peroxidation of membrane lipids and, together with other antioxidants, plays an important role in maintaining cellular integrity (3). As the determinant of the sulfhydryl/disulfide ratio (4), glutathione modulates the activity of a number of enzymes (5), and recently it has been proposed that glutathione may be involved in the transport of amino acids across cell membranes (6). Although this hypothesis is mainly based on in vitro experiments, a role for glutathione in amino acid metabolism in vivo has been supported by recent findings in Meister's laboratory (7) and in our own (8).

Despite the fundamental role of glutathione in such critically important cellular functions, however, relatively little is known about the control mechanisms maintaining glutathione homeostasis in vivo. Most studies have been limited to the measurement of glutathione concentrations under given experimental conditions and reveal little about the kinetic aspects of glutathione metabolism. For example, it has long been known that stress and fasting are associated with decreased hepatic glutathione concentrations (9, 10). The depletion of glutathione during fasting is of interest because fasting is associated with an increase in the hepatic necrosis caused by some electrophilic drug metabolites capable of alkylating nucleophilic sites on vital hepatic macromolecules (11).

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The decreased glutathione concentrations during fasting have generally been considered the result of decreased glutathione synthesis due to a lack of precursor amino acids (12). Indeed, the hepatic concentration of cysteine, which is normally (13) in the range of the Michaelis constant (K_m) of the first and rate-limiting enzyme in glutathione synthesis, gamma-glutamylcysteine synthetase, drops further during starvation (12). However, the decrease in free glutathione during fasting has been shown to be associated with an increase in glutathione-protein mixed disulfides (14). A similar shift in the free glutathione to glutathione/mixed disulfide ratio has been proposed to be responsible for the diurnal variation of free hepatic glutathione, and both the diurnal and the fasting-induced changes correlate with alterations in hepatic cyclic adenosine monophosphate (cAMP) concentrations (14, 15). These observations suggest that a decreased glutathione synthesis may not be the main mechanism responsible for the decreased free glutathione during fasting. In fact, recent data on the possible role of glutathione in the transport of amino acids would lead one to speculate that the synthesis of glutathione might be increased, rather than decreased, during fasting. Short-term starvation is associated with an increase in gamma-glutamyl transpeptidase activity, the major enzyme involved in the catabolism of glutathione (12). Moreover, fasting results in the mobilization of amino acids for gluconeogenesis with an increased uptake of amino acids into the liver, and we have shown recently that administration of amino acids increases hepatic glutathione turnover and consumption (8). During fasting, therefore, the increased load of amino acids and the increased gamma-glutamyl transpeptidase activity should result in an increased glutathione consumption. Consequently, the increased consumption should lead to an increased synthesis of glutathione because, at least in vitro, glutathione synthesis appears to be regulated by feedback inhibition (13).

Analysis of the control mechanisms maintaining glutathione homeostasis in vivo is further complicated by the observation that glutathione concentrations do not drop further as fasting continues but rather reach a nadir of ~70% of control values within 24 h, which is maintained (16, 17). This observation has been construed as evidence for the presence of two glutathione pools, one pool with a fast turnover that is virtually abolished by fasting and a pool with a slow turnover that is not affected by starvation. This interpretation appeared to be supported by the finding of a biexponential decline of the specific activity of glutathione as measured in liver homogenates after administration of radiolabeled amino acid precursors of glutathione (18). However, these investigators failed to account for the delayed contribution to glutathione synthesis made by the recirculation of tracer derived from protein breakdown. A fraction of the radioactive amino acid used to label the glutathione pool will be incorporated into proteins which, as they are metabolized, will provide a radiolabeled amino acid pool for glutathione synthesis. Because overall protein turnover is much slower than glutathione turnover, the time-course of the specific activity of the protein will determine the decline of the specific activity of glutathione during later time periods. Indeed, the specific activity of glutathione more or less paralleled the specific activity of the liver proteins in the studies of Higashi et al. (18). Accordingly, the observed slowly exchangeable pool of glutathione in these studies was most likely an artifact due to recirculation of the radiolabeled amino acid during protein turnover.

Thus, current views on the regulation of glutathione synthesis and the distribution of intracellular glutathione into different pools are difficult to reconcile with some of the more recent experimental data that indirectly reflect aspects of glutathione homeostasis. Accordingly, we have measured the synthesis of glutathione directly during fasting and have attempted to characterize determinants of glutathione turnover in vivo. In addition, we have experimentally tested the hypothesis that several intracellular pools of glutathione exist. For these purposes we have developed an approach that is well suited for the study of acute changes in glutathione kinetics and allows an assessment of the rate of hepatic glutathione turnover in individual animals (19). A small dose of acetaminophen is administered as a pharmacologic probe and the specific activity of previously radiolabeled hepatic glutathione is determined by measurement of the specific activity of the glutathione-acetaminophen adduct in bile or the acetaminophen mercapturic acid in urine.

METHODS

Male Sprague-Dawley rats, weighing 180–230 g (Timco Breeding Laboratories, Houston, Tex.) had free access to food and water and were kept in an air-conditioned room with a controlled 12-h dark-light cycle. For the determination of glutathione turnover the common bile duct and a femoral vein were cannulated under light ether anesthesia and the animals were then restrained. Each turnover study was started between 10:00 and 12:00 a.m. at least 1 h after the animals had awakened from anesthesia. Approximately 15 μCi ^{35}S-L-cysteine (121 mCi/mmol, Amersham Corp., Arlington Heights, Ill.) were administered intravenously followed 10 min later by 50 mg of acetaminophen per kilogram. Other animals received L-[G-^{3}H]glutamic acid (27 Ci/mmol, Amersham Corp.) instead of ^{35}S-cysteine. Because of the short half-life of acetaminophen in rats, repeat doses of acetaminophen (20 mg/kg) were injected 2, 3, and 4 h after the radioactive cysteine. Bile was collected in 20–30-min periods starting 60 min after the injection of the radioactive precursor. The specific activity of the glutathione-acetaminophen adduct appearing in bile was determined by high-pressure liquid chromatography and scintillation spectrometry as described previously (19). The fractional rate of glutathione turnover was calculated

from the monoexponential decline of the specific activity of the glutathione-acetaminophen adduct by least square regression analysis. Extensive studies with different radioactively labeled precursor amino acids of glutathione for measurements of glutathione turnover have shown that this method provides a valid assessment of glutathione turnover in vivo and that the small doses of acetaminophen used as a pharmacologic probe do not stimulate glutathione turnover (19).

To study the influence of fasting on glutathione turnover, rats having free access to water were starved for 48 h prior to the kinetic study. Additional fasted animals were refed for 4 h and glutathione turnover was determined 2 h later. The refed animals ate an average of 6.9 g of Rat Chow prior to the turnover study. At the end of the experiments the liver was removed and hepatic glutathione concentration was determined. To ensure that the hepatic concentration of glutathione remained stable during the determination of turnover, hepatic glutathione was measured in additional rats at the start of the experiment and again 4 h later.

Because diurnal and fasting-induced changes in hepatic glutathione content have been attributed to alterations in cAMP concentration, the effect of cAMP on glutathione turnover was studied in another group of rats. Dibutyryl adenosine 3′,5′-cyclic monophosphoric acid, 50 mg/kg (Sigma Chemical Co., St. Louis, Mo.), and theophylline, 20 mg/kg (Sigma Chemical Co.), were administered subcutaneously to fed rats 3 h after labeling the glutathione pool with ^{35}S-cysteine. The specific activity of the glutathione-acetaminophen adduct in bile was determined as in the previous experiments before and after the injection of cAMP.

In order to assess the physiologic role of cAMP in regulating the turnover of glutathione during fasting, 1.0 g/kg of α-D-glucose dissolved in 2 ml of water was instilled through a duodenal catheter 2.5 h after labeling the glutathione pool in a group of fasted rats. The decreased glucagon/insulin ratio resulting from the administration of glucose decreases the hepatic cAMP concentration (20–22). The turnover of glutathione was measured in the same animal before and after the administration of glucose.

In order to compare the fractional rate of hepatic glutathione turnover of fasted animals with the rate constants of fed rats with comparable hepatic glutathione concentrations a group of fed rats received 1.0 g/kg of diethyl maleate subcutaneously 1 h after labeling the glutathione pool with radioactive cysteine. Hepatic glutathione concentrations reach a nadir 1 h after the administration of diethyl maleate, and the low concentrations of glutathione are maintained for about 2.5 h (23). The rate of hepatic glutathione turnover was, therefore, determined between one and 2.5 h after the injection of diethyl maleate when steady-state conditions may be expected. Indeed, the analysis of the time-course of the specific activities of the glutathione-acetaminophen adduct in bile showed a monoexponential decline of the specific activities during this interval (19).

To determine the size of the glutathione pool probed by the described approach, the specific activity of the glutathione-acetaminophen adduct in bile and the specific activity of the free intracellular glutathione were determined simultaneously in a group of rats. Identical specific activities would indicate that the acetaminophen probe analysis measures the turnover of the total free glutathione pool as measured by chemical analysis, whereas lower specific activities of the hepatic glutathione would indicate the presence of an additional pool of free glutathione that turns over more slowly. The specific activity of the glutathione-acetaminophen adduct in bile samples obtained immediately prior to death was therefore compared with the specific activity of the glutathione in liver homogenates of the same animal.

Analytical methods. To determine the specific activity of the acetaminophen-glutathione adduct in bile, the biliary metabolites of acetaminophen were separated on a C_{18} μBondapak column (Waters Associates Inc., Milford, Mass.) with water/methanol/acetic acid (86.5:12.5:1), 1 ml/min, as the mobile phase (24). The mass of acetaminophen-glutathione was calculated from a standard curve obtained from [^{3}H]-acetaminophen-glutathione collected in the bile of a rat that had received [^{3}H]acetaminophen of known specific activity. The radioactivity in the collected acetaminophen-glutathione peak was determined by liquid scintillation spectrometry with quench correction by the channels ratio technique.

Because this system did not fully resolve the acetaminophen-glutathione adduct from radiolabeled diethyl maleate metabolites, the bile samples of diethyl maleate treated rats were chromatographed with water/methanol/acetic acid (91:8:1) containing 0.005 M heptanesulfonic acid, 1.7 ml/min, as the mobile phase. A comparison of bile from rats that had received either diethyl maleate or acetaminophen in addition to radiolabeled cysteine verified that the chromatographic system resolved the glutathione or cysteine-containing diethyl maleate metabolites from the acetaminophen-glutathione adduct (19).

To determine the specific activity of intrahepatic glutathione, a portion of the liver was homogenized in 5% perchloric acid and the supernate was treated with iodoacetic acid to form *S*-carboxymethyl glutathione. This adduct was conjugated with 1-fluoro-2,4-dinitrobenzene and isolated by high pressure liquid chromatography on a C_{18} μBondapak column using methanol/water/acetic acid (17.5:81.5:1) at a flow rate of 1 ml/min. This system separates the corresponding derivatives of cysteine, cystine, glutathione disulfide, and the cysteine and glutathione adduct of acetaminophen from glutathione as determined by standard compounds derivatized with the identical procedure. Mass was calculated by comparison of the glutathione derivative peak with a standard curve obtained with the synthetic glutathione derivative. The radioactivity of the glutathione derivative was measured by liquid scintillation spectrometry with quench correction by the channels ratio technique.

The concentration of acid soluble sulfhydryls in liver homogenates [20% in 0.1 M phosphate buffer pH 7.4, and 2 mM EDTA] was determined according to Ellman (25) following precipitation of proteins with an equal volume of 4% sulfosalicylic acid. Glutathione disulfide was measured by the method of Tietze following derivatization of glutathione with 2-vinylpyridine (26, 27). The concentration of mixed disulfides was calculated from the difference in acid soluble sulfhydryls before and after incubation of liver homogenates with an equal volume of 2% sodium borohydride in water at 40° for 30 min (28). Foaming was prevented by adding 50 μl *n*-octanol.

Gamma-glutamyl transpeptidase activity was assessed with gamma-glutamyl-nitroanilide as the substrate. The incubation mixture [1 ml] contained 0.05 M Tris-HCl buffer [pH 8], 75 mM NaCl, 2 mM gamma-glutamyl-*p*-nitroanilide, 20 mM glycyl-glycine [pH 8] and 100 μl liver homogenate (29). L-Serine [5 mM] and borate [5 mM] was added to some samples to inhibit the activity of the enzyme. After 1 h at room temperature 1 ml of 1.8 N acetic acid was added to stop the reaction and the absorbance was determined at 405 nm. To demonstrate that increased gamma-glutamyl transpeptidase activity is associated with increased degradation of glutathione the hydrolysis of glutathione catalyzed by liver homogenates was measured in vitro. The assay mixture (2 ml) contained 0.1 M phosphate buffer, pH 7.4, 2 mM EDTA, 3 mM glutathione, and 1 ml of liver homogenate. The glutathione concentration was measured before and after incuba-

tion at 37°C for 3 h by the enzymatic method of Tietze (26). The proteins in liver homogenates were determined by the method of Lowry (30).

Statistical analysis and calculations. All values are expressed as mean±SD. The statistical significance of differences between the experimental groups and between regression coefficients was calculated by Student's *t* test (31). A two-compartment model was used to simulate the time-course of the concentration of tracer in the hepatic glutathione pool following introduction of the tracer into the hepatic pool of one of the precursor amino acids of glutathione (Fig. 1). The tracer was introduced into the precursor compartment (cysteine) at time zero and numerical solution of the system of differential equations were obtained by the Runge-Kutta method (32). Under steady-state conditions the amount of cysteine entering the glutathione pool must equal the amount of cysteine leaving the glutathione pool. Thus, k_{21} (I) = (k_{32} + k_{12}) (II); because the size of the glutathione pool is ~24 times larger than the size of the cysteine pool (12) k_{21} = 24 (k_{32} + k_{12}). The experimentally determined rate constants for the turnover of glutathione in the fed and fasted state were chosen for (k_{32} + k_{12}). In the recirculation model it was assumed that 50% of the glutathione turnover is accounted for by efflux of glutathione from the liver. The arbitrary choice of k_{41} is based on the assumption that only a fraction of the available cysteine will be used for glutathione synthesis.

RESULTS

As shown for a group of refed and starved rats in Fig. 2, the specific activity of the glutathione-acetaminophen adduct in bile declined monoexponentially from 1 h onward after the injection of radiolabeled cysteine or glutamic acid. The fractional rate of glutathione turnover was calculated by linear regression analysis after logarithmic transformation of the data, as previously reported (19). During fasting the rate constant for hepatic glutathione turnover was much higher than in refed rats of the same age or in animals that had free access to food prior to the experiment (Table I). Quantitatively

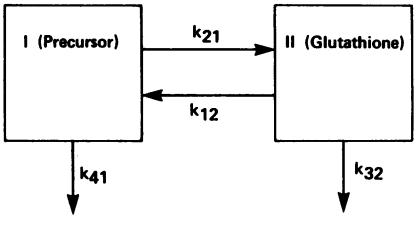

FIGURE 1 Compartment model used to simulate the time-course of the concentration of tracer in the hepatic glutathione pool (II) following introduction of the tracer into the hepatic pool of one of the precursor amino acids of glutathione (I). k_{21} is the fraction of the precursor pool entering the glutathione pool per unit time; k_{41}, the fraction entering other metabolic pathways of the precursor amino acid such as protein synthesis. k_{12} plus k_{32} equals the fractional rate of turnover of the glutathione pool. k_{12} is the fraction of the labeled amino acid in the glutathione pool reentering the precursor pool following the catabolism of glutathione; k_{32}, the fraction of the labeled amino acid in the glutathione pool lost to the precursor pool due to efflux of intact glutathione.

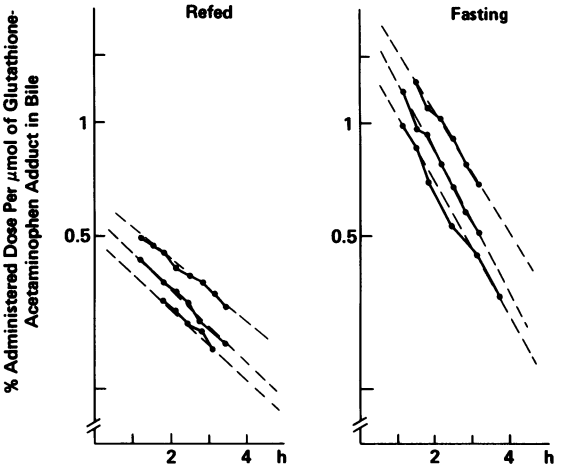

FIGURE 2 Time-course of the specific activity of the glutathione-acetaminophen adduct in bile of rats fasted for 48 h or refed for 4 h after a 48-h fast. Each curve represents data obtained from an individual animal. The radioactively labeled glutathione precursor ^{35}S-L-cysteine was administered at time zero and small doses of acetaminophen were injected intravenously at intervals to trap the glutathione as an excretable adduct. The fractional rate of glutathione turnover was calculated by linear regression analysis after logarithmic transformation of the specific activities.

similar increases in the rate constant were observed following the administration of either labeled cysteine or glutamic acid, which indicates that the measured rate constant indeed reflects the fractional turnover of glutathione itself and not the turnover of the administered precursor amino acid. Due to the increased rate of turnover and the decreased size of the hepatic glutathione pool, fasting animals incorporated a higher fraction of the administered precursor amino acid per micromole glutathione than refed animals, as shown by the higher specific activities of the adduct. The incorporation of the precursor amino acid and the rate constant were not significantly different in the refed animals and in non-fasted animals receiving a regular diet (Table I).

A comparison of the simultaneously determined specific activities of free hepatic glutathione and of the glutathione-acetaminophen adduct in bile is shown in Fig. 3. If two kinetically distinct intrahepatic pools of glutathione were present, the specific activity of the glutathione in each pool would be different except for one point in time when the two specific activity time curves intersect. To ensure that the specific activity of the free glutathione and the glutathione-acetaminophen adduct were not accidentally measured at that particular point in time the animals were killed at different times after the administration of the labeled precursor amino acid. This experimental design results in the wide range of specific activities shown in Fig. 3. The specific activity of the free hepatic glutathione averaged 98±11% (n = 10) of the specific activity of the

TABLE I

Effects of Fasting and Refeeding on the Fractional Rate of Hepatic Glutathione Turnover and Glutathione Synthesis

Regular diet			Refed for 4 h following 48-h fast			Fasting for 48 h		
K	GSH*	GSH Synthesis	K	GSH*	GSH Synthesis	K	GSH*	GSH Synthesis
h^{-1}	μmol/g	μmol/g per h	h^{-1}	μmol/g	μmol/g per h	h^{-1}	μmol/g	μmol/g per h
0.258	4.8	1.24	0.319	4.0	1.28	0.589‡	2.0	1.18
0.240	3.5	0.84	0.317	4.0	1.27	0.492‡	2.5	1.23
0.229‡	4.8	1.10	0.289	2.4	0.69	0.474	2.9	1.37
0.198‡	3.6	0.71	0.204	6.2	1.26	0.468	4.3	2.01
0.186	3.9	0.73	0.174	6.8	1.18	0.444	3.8	1.69
0.185	3.9	0.72	0.174	5.4	0.94	0.444‡	3.2	1.42
0.179	4.9	0.88				0.415	4.1	1.70
0.174‡	5.3	0.92				0.406‡	3.6	1.46
0.150‡	5.8	0.87				0.357	4.0	1.43
0.126	6.1	0.77				0.352	4.7	1.65
0.124	5.6	0.69				0.310	4.3	1.33
0.186±0.042	4.7±0.9	0.86±0.17	0.246±0.064	4.8±1.5	1.10±0.22	0.432±0.074§	3.6±0.8‖	1.50±0.23¶

* GSH, hepatic glutathione concentration determined by the Ellman method prior to reduction of disulfides with borohydride.

‡ These animals received [^{3}H]glutamic acid instead of cysteine to label the glutathione pool.

§ Significantly different ($P < 0.001$) from regular diet and refed groups.

‖ Significantly different ($P < 0.01$) from regular diet groups.

¶ Statistically different from regular diet ($P < 0.001$) and refed ($P < 0.01$) groups.

glutathione-acetaminophen adduct in bile. This observation provides strong evidence that our approach probes the same glutathione pool as the chemical analysis of the free intrahepatic glutathione. During the 4-h interval required to measure the rate constant of glutathione turnover, the glutathione concentration remained virtually constant, decreasing by an average of only 0.38 and 0.28 μmol/g in three pairs of fed and fasted animals, respectively. Therefore, the synthesis of glutathione can be estimated by multiplying the chemically determined free glutathione concentration at the end of the experiment with the measured fractional rate of turnover. Based on these calculations (Table I) the estimated synthesis of glutathione was significantly higher during fasting than in control and refed animals.

The decreased concentration of glutathione during fasting that occurs together with an increase in glutathione synthesis suggests an increased consumption of glutathione in the fasting state. Because gamma-glutamyl transpeptidase is the main enzyme involved in the degradation of glutathione its activity was measured in liver homogenates of fed and fasted animals. Using gamma-glutamyl-nitroanilide as the substrate and glycyl-glycine as the acceptor for the glutamyl moiety, the gamma-glutamyl transpeptidase activity increased from 3.47±0.80 [$n = 6$] μmol nitroanilide released/h per g liver in fed rats to 5.67±1.72 μmol/h per g [$P < 0.05$] after a fast of 48-h duration. Addition of serine-borate to the incubation mixture abolished the release of nitroanilide. When liver homogenates were incubated with glutathione alone the homogenates of fed rats hydrolyzed 0.28 ±0.21 μmol glutathione/h per g liver compared to 2.29±1.06 μmol/h per g [$P < 0.05$] in fasted animals. The relative difference in these activities between fed and fasted rats does not change if expressed per milli-

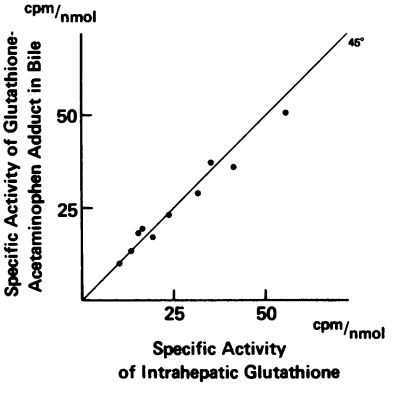

FIGURE 3 Comparison of the simultaneously determined specific activities of free hepatic glutathione and the glutathione-acetaminophen adduct in bile. Samples were obtained at different time intervals following the administration of the labeled precursor amino acid. The virtual identity of the specific activities of the adduct and the free glutathione over a wide range of specific activities indicates that no sizeable additional pool of glutathione with a slow rate of turnover is present within the liver.

gram protein instead of per gram liver, because the protein content per gram liver was identical in fed and fasted animals.

During fasting the percentage of the total glutathione (measured following incubation with borohydride) present as mixed disulfides increased from 31.9±5.0% to 53.9±2.9% [$P < 0.01$]. The concentration of glutathione disulfide (0.072±0.015 mM), however, did not change. A similar shift in the free sulfhydryl to mixed disulfide ratio during fasting has been shown to correlate with increases in the intracellular concentration of cAMP and has been reproduced by the administration of exogenous dibutyryl cAMP (14). Moreover cAMP has been shown to increase the activity of the glutathione catabolic enzyme gamma-glutamyl transpeptidase (33). From these observations, cAMP would therefore be expected to increase the rate of turnover of glutathione. To test this hypothesis, dibutyryl cAMP and theophylline were administered to fed animals and hepatic glutathione turnover was determined (Fig. 4). This treatment promptly increased the slope of the specific activity time curve, indicating an increased influx of unlabeled cysteine into the glutathione pool. The similar effect of exogenous cAMP and fasting on the rate at which the specific activity of glutathione declines, suggests that increased concentrations of cAMP during fasting may be responsible for the altered glutathione kinetics.

To test whether this effect of exogenous cAMP reflects a possible physiologic response to the increased concentrations of cAMP stimulated by glucagon release during fasting, an attempt was made to reverse the fasting-induced increase in the measured rate constant of glutathione turnover by decreasing hepatic cAMP concentrations. To decrease the glucagon: insulin ratio and thus the hepatic concentration of cAMP (Fig. 5), glucose was administered intraduodenally 2.5 h after labeling the glutathione pool in fasted rats. The glucose load promptly reduced the slope of the specific activity time curve of the glutathione-acetaminophen adduct.

The association of an increased fractional rate of glutathione turnover with decreased levels of free glutathione observed during fasting might be the result of feedback regulation of glutathione synthesis. Fig. 6 demonstrates that as the hepatic glutathione concentration was reduced by the administration of diethyl maleate to fed animals, the fractional rate of hepatic glutathione turnover indeed increased. However, the fractional rate of turnover measured in fasted animals was clearly higher than in fed, diethyl maleate-treated animals with comparable glutathione levels and thus resulted in a greater amount of glutathione synthesized during fasting.

The simulated data (Fig. 7) demonstrate that in agreement with the experimental data the peak specific

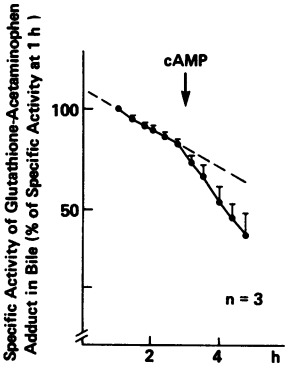

FIGURE 4 Effect of dibutyryl cAMP and theophylline on the time-course of the specific activity of the glutathione-acetaminophen adduct in bile. Each point represents mean ±SD of three studies in three individual animals. Because of variable doses of radioactivity the initial specific activities were not identical in all studies. The specific activities are, therefore, expressed as a percentage of the specific activity at 1 h. Subcutaneous administration of cAMP and theophylline results in an increased slope of the specific activity time curve. The slopes before and after cAMP are significantly different ($P < 0.05$).

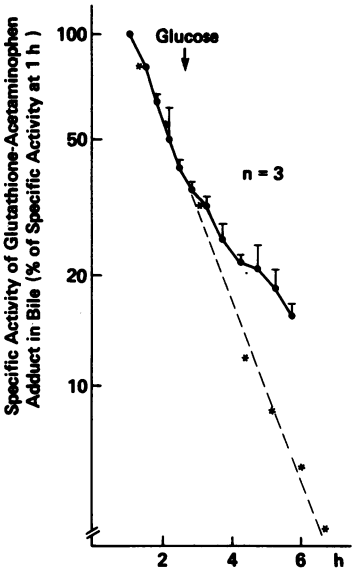

FIGURE 5 Effect of intraduodenally administered glucose on the time-course of the specific activity of the glutathione-acetaminophen adduct in bile of rats that had been fasted for 48 h. Each point represents mean ± SD of three studies in three individual animals. Because of variable doses of radioactivity the initial specific activities were not identical in all studies. The specific activities are, therefore, expressed as a percentage of the specific activity at 1 h. Administration of glucose reduces the slope of the specific activity time curve. The slopes before and after glucose are significantly different ($P < 0.05$). The specific activity time curve obtained in a fasted rat with a similar initial rate of turnover is shown for comparison (*).

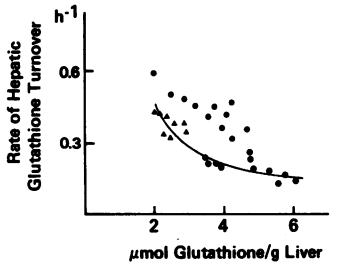

FIGURE 6 Rate of hepatic glutathione turnover as a function of the intrahepatic glutathione concentration. The fractional rate of glutathione turnover increases with decreasing concentrations of glutathione in fed rats (●) and fed rats pretreated with diethyl maleate (▲). Fasting animals (○) have a higher fractional rate of glutathione turnover than animals with comparable intrahepatic glutathione concentrations.

activity of the glutathione pool will be higher in the fasted than in the fed state and that the specific activity of cysteine will decrease at an only slightly higher rate than the specific activity of glutathione after 1 h if a fraction of the tracer recirculates. The simulated data also show that the experimentally determined rate constant of the turnover of glutathione will underestimate the actual rate constant if a fraction of the tracer recirculates, such as might occur from recirculation of tracer originating from catabolism of protein or glutathione.

DISCUSSION

The presented data demonstrate that hepatic glutathione synthesis during fasting is increased and not decreased as generally assumed. For the determination of the rate of glutathione synthesis by the current approach, several criteria must be fulfilled. First, the calculation of glutathione synthesis from the intrahepatic concentration and from the fractional rate of turnover is obviously valid only if the pharmacologic probe analyzes the total glutathione pool measured by the chemical analysis of glutathione by the Ellman method; it would not be accurate if two hepatic pools with different rates of turnover were present. Our experiments, however, failed to demonstrate the presence of two glutathione pools. Because our acetaminophen probe analysis yields glutathione half-lives corresponding to the proposed glutathione pool with rapid turnover (18), the specific activity of the intrahepatic glutathione should be lower than the specific activity of the glutathione-acetaminophen adduct if a sizeable pool with slow turnover were present. However, the specific activities of the free glutathione in liver homogenates and the glutathione-acetaminophen adduct in bile were similar, indicating that the total free glutathione pool is kinetically homogeneous and therefore the calculation of hepatic glutathione synthesis from the turnover

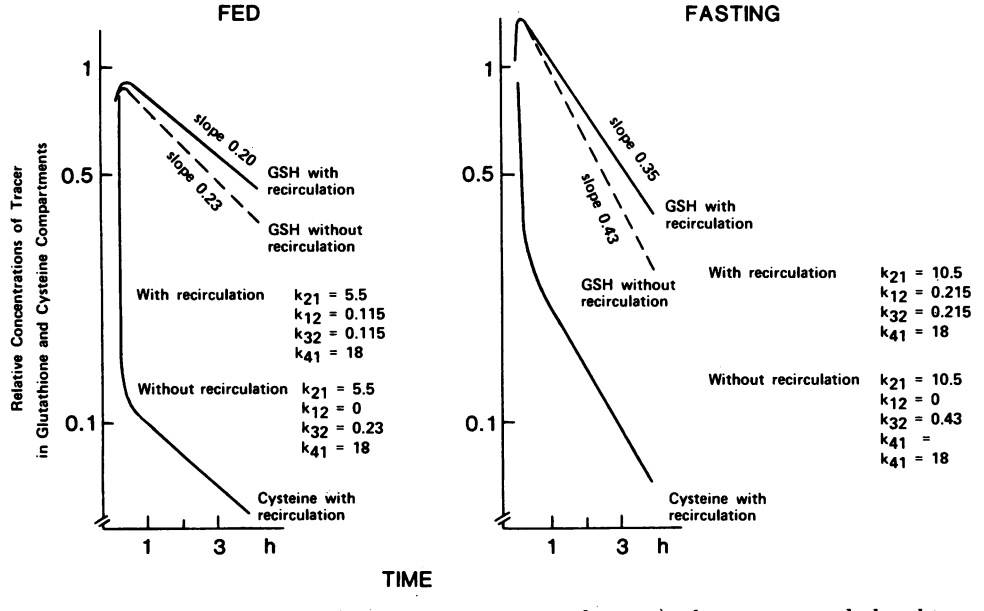

FIGURE 7 Time-course of the relative concentrations of tracer in the cysteine and glutathione pool using the model shown in Fig. 1 and the rate constants indicated in the figure. The simulation, in agreement with the experimental data, demonstrates that the peak specific activity of the glutathione pool will be higher in the fasted than in the fed state and that the specific activity of cysteine will decrease at an only slightly higher rate than the specific activity of glutathione after 1 h if a fraction of the tracer recirculates. The simulated data also show that the experimentally determined rate constant of the turnover of glutathione will underestimate the actual rate constant if a fraction of the tracer recirculates, such as might occur from recirculation of tracer originating from catabolism of protein or glutathione. GSH, hepatic glutathione concentration.

rate constant and pool size is appropriate. In support of this, Griffith and Meister have recently come to similar conclusions (34) by a different approach. In their studies, inhibition of glutathione synthesis in vivo resulted in a monophasic decline of intrahepatic glutathione to 20% of control values, a finding that speaks against the presence of separate pools of glutathione with different turnover rates.

The other major consideration for the determination of glutathione synthesis is the precursor-product relationship that exists between an amino acid precursor and glutathione, such that changes in the kinetics of the precursor pool caused by fasting could artifactually affect the experimentally determined rate constant for turnover of the glutathione pool. Because fasting leads to a decrease in the intrahepatic concentration of cysteine (12), fasting may be particularly prone to alter the kinetics of the cysteine pool. To understand better the influence of changes in the precursor kinetics on the experimental data obtained by acetaminophen probe analysis, a model was designed for analysis of the time-course of the tracer through the precursor and glutathione compartments under various conditions (Figs. 1 and 7). The simulated data show that the initial alpha phase of the biexponential decline of the specific activity of cysteine can not affect the measured rate constant for glutathione turnover because it declines at a much greater rate than the fractional rate of glutathione turnover. On the other hand, the terminal beta phase, which is mainly determined by recirculation of tracer, can markedly affect the measured rate constant for glutathione turnover. Because the cysteine pool is decreased during fasting, it is conceivable that increased recirculation of tracer resulting from increased breakdown of glutathione by gamma-glutamyl transpeptidase could occur during fasting and lead to an underestimation of the actual fractional turnover of glutathione. Increased recirculation of cysteine resulting from increased protein turnover would have a similar effect. In contrast to cysteine, however, the size of the glutamic acid pool is much larger and does not decrease during fasting (12) and thus should be minimally affected by food deprivation. Accordingly, the quantitatively similar increase in the rate constant for glutathione turnover produced by fasting when labeled glutamic acid is used as the glutathione precursor indicates that changes in the kinetics of the precursor pool during fasting do not significantly influence the fractional rate of glutathione turnover and therefore our estimates of glutathione synthesis.

Studies in vitro have documented that glutathione regulates its own synthesis by feedback inhibition of the rate limiting step, the formation of gamma-glutamyl-cysteine by gamma-glutamyl-cysteine synthetase (13). Our observation that a decrease in glutathione concentration is accompanied by an increase in the rate of turnover of glutathione demonstrates that a similar feedback regulation of glutathione synthesis is operative in vivo (Fig. 6). It is, therefore, not surprising that the decrease in free hepatic glutathione during fasting is associated with an increased fractional rate of glutathione turnover.

Our data, however, also indicate that the measured increase in the fractional rate of glutathione turnover and hence the synthesis of glutathione is greater in fasted animals even than in fed, diethyl maleate-treated rats with comparable glutathione concentrations (Fig. 6). Under the steady-state conditions that may be expected once the new plateau of glutathione is reached during fasting, this increased synthesis of glutathione must reflect an increased consumption of glutathione. The major route of glutathione catabolism is through the gamma-glutamyl cycle, which may be involved in the transport of amino acids across cell membranes (6). As shown above, the consumption of glutathione and the activity of the enzyme gamma-glutamyl transpeptidase, which is responsible for the degradation of glutathione via the gamma-glutamyl cycle, are markedly increased in homogenates from fasted animals, confirming the previous report by Tateishi et al. (12). These data obtained in vitro thus are consistent with the interpretation from our in vivo kinetic data that the consumption of glutathione increases during fasting.

The quantitative contribution of this catabolic pathway to the turnover of hepatic glutathione is not clear. However, we have recently shown that the intravenous administration of methionine and several other amino acids increase the rate of turnover and decrease the concentration of hepatic glutathione, and this stimulation was abolished by the specific gamma-glutamyl transpeptidase inhibitor, gamma-glutamyl carboxy phenylhydrazine (8). Since fasting results in a mobilization of amino acids for hepatic gluconeogenesis and stimulates hepatic amino acid uptake (35), an increased transpeptidation of the amino acids via the gamma-glutamyl cycle might well explain the increased consumption of glutathione observed in fasted animals.

The present data also provide strong support for the hypothesis (14, 33) that the fasting-induced decrease in the hepatic free glutathione/mixed disulfide ratio and the increase in glutathione consumption results from an increase in cAMP content. The administration of dibutyryl cAMP and theophylline promptly stimulates glutathione turnover (Fig. 4). A similar effect of cAMP can be inferred from the data of Higashi et al. (33) who found a lower specific activity of hepatic glutathione in liver homogenates after administration of dibutyryl cAMP, a result that would be expected if cAMP increased glutathione turnover. That the effect of cAMP is not purely pharmacologic but rather physiologic is suggested by our experiments where glucose was administered intraduodenally and promptly slowed the rate of glutathione turnover. The glucose

load may be expected to decrease the glucagon/insulin ratio, thereby decreasing the hepatic concentration of cAMP (21, 22). Consequently, the fasting-induced decrease in the glutathione/mixed disulfide ratio is reversed, and the concentration of free glutathione increases. In order to maintain glutathione synthesis, the expanded pool thus will have to be renewed less rapidly. However, other mechanisms might possibly contribute to the decrease in the slope of the specific activity time curve, such as the release of glutathione of a higher specific activity from mixed disulfides. This would have the same effect on the measured fractional rate of turnover as an increased recirculation of tracer (Fig. 7) and result in an underestimation of the actual rate constant.

These studies do not address the mechanisms by which cAMP decreases the concentration of free glutathione and increases the activity of gamma-glutamyl transpeptidase. Recent data indicate that cAMP inhibits catalase and, thus, could lead to an increased oxidation of glutathione to glutathione disulfide by endogenous peroxides via glutathione peroxidase (14). As a consequence of its metabolic effects cAMP may also decrease the availability of reducing equivalents required to reduce glutathione disulfide. Both mechanisms result in an increase in glutathione disulfide and, as a consequence of disulfide exchange, in a decreased free glutathione/protein-mixed disulfide ratio.

One other point emerges from the current experiments and merits discussion. The administration of large doses of drugs such as acetaminophen results in the excretion of large quantities of the hepatotoxic metabolite of the drug as a glutathione adduct, which appears in the urine as a mercapturic acid congener. In fact, the quantity excreted can exceed by several-fold the amount of glutathione initially present in the liver (36, 37). Since necrosis occurs only after the availability of glutathione is exhausted (38) the liver must be able to synthesize rapidly great quantities of glutathione in order to prevent cell damage. Therefore, fasting may be a double hazard for liver injury by such compounds. First, less free glutathione is present in the liver for detoxification of the reactive drug intermediates. Second, glutathione synthesis is already markedly stimulated during fasting. Thus, the ability of the liver to synthesize the additional amounts of glutathione required for detoxification of electrophilic metabolites is compromised.

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