Selenium deficiency and vitamin E deficiency both affect xenobiotic metabolism and toxicity. In addition, selenium deficiency causes changes in the activity of some glutathione-requiring enzymes. We have studied glutathione metabolism in isolated hepatocytes from selenium-deficient, vitamin E-deficient, and control rats. Cell viability, as measured by trypan blue exclusion, was comparable for all groups during the 5-h incubation. Freshly isolated hepatocytes had the same glutathione concentration regardless of diet group. During the incubation, however, the glutathione concentration in selenium-deficient hepatocytes rose to 1.4 times that in control hepatocytes. The selenium-deficient cells also released twice as much glutathione into the incubation medium as did the control cells. Total glutathione (intracellular plus extracellular) in the incubation flask increased from 47.7 ± 8.9 to 152 ± 16.5 nmol/10^6 selenium-deficient cells over 5 h compared with an increase from 46.7 ± 7.1 to 92.6 ± 17.4 nmol/10^6 control cells and from 47.7 ± 11.7 to 79.5 ± 24.9 nmol/10^6 vitamin E-deficient cells. This overall increase in glutathione concentration suggested that glutathione synthesis was accelerated by selenium deficiency. The activity of γ-glutamylcysteine synthetase was twice as great in selenium-deficient liver supernatant (105,000 x g) as in vitamin E-deficient or control liver supernatant (105,000 x g). Hemoglobin-free perfused livers were used to determine the form of glutathione released and its route. Selenium-deficient livers released 4 times as much GSH into the caval perfusate as did control livers. Plasma glutathione concentration in selenium-deficient rats was found to be 2-fold that in control rats, suggesting that increased GSH synthesis and release is an in vivo phenomenon associated with selenium deficiency.

Selenium deficiency and vitamin E deficiency both affect xenobiotic metabolism and toxicity (1, 2). Attempts to relate these effects to known biochemical functions of the nutrients have not usually been successful. In the case of selenium, for example, the only known animal selenoenzyme is glutathione peroxidase, but we have dissociated the activity of this enzyme from the protective effect of selenium against diquat-induced hepatotoxicity and lipid peroxidation (3). The mechanisms by which vitamin E deficiency potentiates acetaminophen hepatotoxicity in the rat and selenium deficiency ameliorates it are also unexplained (4). Since selenium and vitamin E are generally considered to be oxidant defenses, it is particularly intriguing that vitamin E deficiency increased the hepatotoxicity of acetaminophen without a corresponding increase in lipid peroxidation (4).

Glutathione plays a very prominent role in hepatic xenobiotic metabolism and detoxification. While the effects of selenium deficiency and of vitamin E deficiency on glutathione-dependent enzymes have been reported (4, 5), little is known about how these deficiencies influence glutathione synthesis and turnover. The present studies were undertaken with two major aims. One was to determine whether hepatocytes isolated from selenium-deficient rats and from vitamin E-deficient rats would be suitable for studies of drug metabolism and toxicity. The other was to examine glutathione metabolism in these isolated hepatocytes to learn whether any of the effects of selenium or vitamin E might be mediated through alterations in it.

**EXPERIMENTAL PROCEDURES**

**Materials**—Collagenase, class II (145 units/mg) was obtained from Worthington. All amino acids, GSH, GSSG, glutathione reductase, NADPH, and ATP were purchased from Sigma. L-[3H]Glutamic acid was purchased from New England Nuclear. All other chemicals were reagent grade or better.

**Animals**—Livers from male Sprague-Dawley rats (250-450 g) which had been fed the experimental diets from weaning for at least 14 weeks were used in all experiments. The diet was prepared as described previously (6). In the control diet, 0.5 mg/kg of selenium as Na₂SeO₃ and 100 IU/kg of vitamin E as dl-a-tocopheryl acetate were added. The selenium-deficient diet omitted the selenium, and the vitamin E-deficient diet omitted the dl-a-tocopheryl acetate and contained tocopheryl-stripped corn oil. The rats had free access to food and water up to the time of the experiment. Selenium deficiency was verified by measurement of glutathione peroxidase in the livers of some of the rats fed the selenium-deficient diet (7). Vitamin E deficiency was verified by measurement of α-tocopherol in the plasma of some of the rats fed the vitamin E-deficient diet (8).

**Solutions**—All buffer solutions were bubbled with O₂/CO₂ (95:5) and warmed to 37 °C prior to use.

**Solution A:** 112 mM NaCl, 5.6 mM KCl, 1.0 mM NH₄Cl, 1.4 mM KH₂PO₄, 1.5 mM MgSO₄, 29.5 mM NaHCO₃, pH 7.4.

**Solution B:** 119 mM NaCl, 4.7 mM KCl, 0.8 mM NH₄Cl, 1.2 mM KH₂PO₄, 1.3 mM MgSO₄, 24.8 mM NaHCO₃, 48.8 mM lactate, 5.3 mM fumarate, 48.8 mM L-glutamate, 11.4 mM α-D(+)-glucose and amino acid mixture, pH 7.4.

**Solution C:** 118 mM NaCl, 4.7 mM KCl, 1.0 mM NH₄Cl, 1.2 mM KH₂PO₄, 1.2 mM MgSO₄, 24.7 mM NaHCO₃, 2.5 mM CaCl₂, 48.7 mM lactate, 5.3 mM fumarate, 48.7 mM L-glutamate, 11.4 mM α-D(+)-glucose, and amino acid mixture, pH 7.4.

Amino acid mixture (final concentrations of L-amino acids in solutions B and C): 0.43 mM alanine, 0.014 mM D-amino butyric acid, 0.033 mM aspartic acid, 0.19 mM arginine, 0.067 mM asparagine, 0.076 mM citrulline, 0.060 mM cysteine, 0.24 mM glutamine, 0.38 mM glycine, 0.076 mM histidine, 0.095 mM isoleucine, 0.16 mM leucine, 0.38 mM lysine, 0.5 mM methionine, 0.086 mM ornithine, 0.076 mM phenylalanine, 0.17 mM proline, 0.27 mM serine, 0.19 mM threonine, 0.29 mM tryptophan, 0.070 mM tyrosine, 0.19 mM valine.
Hepatocyte Preparation—The rat was anesthetized with pentobarbital (100 g of body weight), the peritoneal cavity was opened; the portal vein was cannulated; and solution A was perfused through the liver. The liver was removed and transferred to the perfusion apparatus (9) and perfusion with solution B was begun. The first 50 ml of solution B perfused through the liver was discarded in order to remove any remaining blood. The perfusion system was then temperature regulated at 37 °C, and the liver was perfused for 30 min with 100 ml of solution B containing 50 mg of collagenase. After the collagenase treatment, the liver connective tissue was well digested and the liver was soft. The liver was removed from the perfusion apparatus and was minced in solution C. With minimal agitation, the hepatocyte suspension was filtered through nylon mesh to remove connective tissue and clumps of cells. The suspension was centrifuged for 1 min at 50 x g, the supernatant was discarded, and the cells were resuspended in solution C. This wash procedure was repeated three times. After the final wash, the hepatocytes were suspended in solution C containing 1.5% gelatin. The cells were then placed in a shaking incubator at 37 °C and maintained in a O2/CO2 (20:80) atmosphere for the duration of the experiment. The final cell concentration was adjusted to 3 x 10^6 cells/ml.

Cell viability was measured by mixing an aliquot of the hepatocyte suspension with an equal volume of solution C containing 1% trypan blue. Cells which excluded trypan blue after 5 min were considered to have intact cell membranes and to be viable (10).

Liver Perfusion—The technique of hemoglobin-free, nonrecirculating liver perfused previously (11) was used with some modifications. The bile duct was cannulated and bile was collected separately from the caval perfusate. The perfusion buffer contained 118 mM NaCl, 5.9 mM KCl, 4.3 mM MgSO4, 1.2 mM Na2SO4, 1.3 mM NaH2PO4, 3.3 mM CaCl2, 25 mM NaHCO3, 21.5 mM lactate, 0.3 mM pyruvate. It was equilibrated with O2/CO2 (95:5) to give a final pH of 7.5. The flow rate was adjusted for all livers to 2.5 ml/g/min. The perfusion buffer was warmed so that the effluent temperature was 37 °C. Lactate dehydrogenase activity (12) was measured in all perfusate samples as an index of cell membrane damage.

Lipid Peroxidation was quantitated by determining the amount of thiobarbituric acid-reactive substances present (13). A 250-µl aliquot of hepatocyte suspension was mixed with 2.0 ml of thiobarbituric acid-trichloroacetic acid solution containing 0.01% butyryl-
ated hydroxylsolvane. The mixture was heated in boiling water for 15 min and was centrifuged at 1500 x g for 10 min. The absorbance of the supernatant was measured at 535 nm. Malonaldehyde bis-(di-
methyl acetal) (Aldrich) was used as a standard.

Total glutathione concentration was measured with the glutathione reductase-DTNB1 recirculating assay by the method of Tietze (14) as modified by Griffith (15). A 1.0-ml sample of hepatocyte suspension was centrifuged in a Beckmann Microfuge B for 10 s. The extracellular glutathione concentration was determined in the supernatant. The cells were deproteinized with 3% metaphosphoric acid. The acid supernatant was neutralized with 0.3 M NaOH and was centrifuged at 1500 g for 10 min. The absorbance of the supernatant was measured at 412 nm. Malonaldehyde bis-(dimethyl acetal) (Aldrich) was used as a standard.

The initial glutathione concentration in the hepatocytes was the same for all the diet groups (Fig. 3, top). As the incubation proceeded, however, the amount of glutathione in the selenium-deficient rats rose to about 1.4 times the control level, while the glutathione content of the vitamin E-deficient hepatocytes remained about the same as that in control hepatocytes. More strikingly, the selenium-deficient hepatocytes released twice as much glutathione into the medium as the control or the vitamin E-deficient hepatocytes released (Fig. 3, bottom). The intracellular glutathione concentration of the vitamin E-deficient hepatocytes whose via-

1 The abbreviation used is: DTNB, 5,5'-dithiobis(2-nitrobenzoic acid).

RESULTS

Isolated hepatocytes from selenium-deficient, vitamin E-deficient and control rats were incubated for 5 h. The hepatocytes from all groups maintained similar levels of viability (Fig. 1) with the exception of those isolated from severely vitamin E-deficient rats. Lipid peroxidation in the cells, as measured by thiobarbituric acid-reactive substances, was low and not significantly different in the three major groups, but it was markedly elevated in the hepatocytes isolated from the severely vitamin E-deficient rats (Fig. 2).

The initial glutathione concentration in the hepatocytes was the same for all the diet groups (Fig. 3, top). As the incubation proceeded, however, the amount of glutathione in the selenium-deficient hepatocytes rose to about 1.4 times the control level, while the glutathione content of the vitamin E-deficient hepatocytes remained about the same as that in control hepatocytes. More strikingly, the selenium-deficient hepatocytes released twice as much glutathione into the medium as the control or the vitamin E-deficient hepatocytes released (Fig. 3, bottom). The intracellular glutathione concentration of the vitamin E-deficient hepatocytes whose via-

FIG. 1. Viability of isolated hepatocytes measured by the percentage of cells which excluded trypan blue, O, control hepatocytes; △, selenium-deficient hepatocytes; ●, vitamin E-deficient hepatocytes; ●, vitamin E-deficient hepatocytes isolated from rats fed the diet at least 21 weeks. Each point represents the mean of at least four experiments and one standard deviation is indicated by the bracket.
Glutathione synthesis occurs in two steps. The first step is γ-glutamylcysteine formation by the action of γ-glutamylcysteine synthetase. γ-Glutamylcysteine synthetase activity in selenium-deficient rat liver was double that in control (Table I). This is further evidence that hepatic glutathione synthesis is increased in selenium deficiency. Vitamin E-deficient livers had slightly less activity than controls. Cysteine concentration is thought to be related to the glutathione synthesis rate. Therefore, the liver cysteine concentration was measured. It was lower in selenium-deficient liver than in control liver (Table II), possibly reflecting its greater utilization in glutathione synthesis. The cysteine concentration in vitamin E-deficient liver was slightly, though not significantly, increased over control. γ-Glutamyltranspeptidase activity was measured in the liver homogenate. There was no activity detected in control or selenium-deficient homogenate.

In order to extend the observations in isolated hepatocytes to the intact liver, glutathione release by isolated perfused livers was measured. Because glutathione is released by the liver into the caval perfusate and into the bile, the bile duct was cannulated and the bile was collected separately from the caval perfusate. The glutathione released into the caval perfusate and that released into the bile was measured. Table III shows that the selenium-deficient livers released approximately 4 times as much glutathione into the caval perfusate as did the control livers. The amount of glutathione released into the bile was the same in both diet groups. Lactate dehydrogenase activity was measured in each sample of the caval perfusate and was less than 1 milliunit/ml.

**Table I**

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>γ-Glutamylcysteine formed* (nmol/mg protein/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7</td>
<td>4.49 ± 0.79b</td>
</tr>
<tr>
<td>Selenium-deficient</td>
<td>7</td>
<td>8.35 ± 1.80b</td>
</tr>
<tr>
<td>Vitamin E-deficient</td>
<td>4</td>
<td>3.46 ± 0.16b</td>
</tr>
</tbody>
</table>

*Values are means ± S.D.

**Values significantly different from each other (p < 0.01) by Duncan’s multiple range test have the same superscript letter (b or c).**

**Table II**

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>Cysteine* (μmol/g liver)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8</td>
<td>0.100 ± 0.021b</td>
</tr>
<tr>
<td>Selenium-deficient</td>
<td>8</td>
<td>0.066 ± 0.024b</td>
</tr>
<tr>
<td>Vitamin E-deficient</td>
<td>5</td>
<td>0.119 ± 0.019b</td>
</tr>
</tbody>
</table>

*Values are means ± S.D.

**Values significantly different from each other (p < 0.05) by Duncan’s multiple range test have the same superscript letter (b or c).**

**Table III**

<table>
<thead>
<tr>
<th>Diet group</th>
<th>n</th>
<th>Total Glutathione, nmol GSH/g liver/min</th>
<th>Oxidized Glutathione, nmol GSH/g liver/min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4</td>
<td>1.41 ± 0.13 0.64 ± 0.08 5.62 ± 0.86b &lt;1.2</td>
<td></td>
</tr>
<tr>
<td>Selenium-deficient</td>
<td>3</td>
<td>1.42 ± 0.17 0.54 ± 0.18 22.8 ± 6.47b &lt;1.2</td>
<td></td>
</tr>
</tbody>
</table>

*Values are mean ± S.D. where the value for each animal is the average of three time points of the perfusion (10, 20, and 30 min). All values are expressed in units of nanomoles of GSH/g of liver/min.

**Values significantly different from each other (p < 0.01) by Duncan’s multiple range test.**

Oxidized glutathione was not detectable in the perfusate by the methods used. The minimum concentration detectable is 1.2 nmol/g of liver/min (expressed as GSH equivalents).
The forms of glutathione released by the perfused livers were determined (Table III). Approximately 40% of the biliary glutathione was in the oxidized form in both diet groups. Oxidized glutathione was not detectable in caval perfusate from either selenium-deficient or control livers, indicating that at least 95% of the glutathione released into the caval perfusate by selenium-deficient livers was in the reduced form. Thus, selenium deficiency accelerated hepatic glutathione synthesis and increased the release of reduced glutathione into the blood.

The increased release of glutathione by the selenium-deficient liver was confirmed by determining plasma glutathione concentrations. As shown in Table IV, plasma from selenium-deficient rats contained 3 times as much glutathione as control plasma. Upon treatment with NaBH₄ to recover any GSH involved in mixed disulfides, the selenium-deficient plasma contained 2 times as much glutathione as control plasma. These data support our conclusion that the increased glutathione release in selenium deficiency is an in vivo as well as an in vitro phenomenon.

**DISCUSSION**

In this study, we have isolated selenium-deficient and vitamin E-deficient hepatocytes and have maintained them in a complete medium for 5 h. With the exception of cells isolated from severely vitamin E-deficient rats, the viabilities of deficient hepatocytes were similar to the viability of control hepatocytes. The cells isolated from rats fed the vitamin E-deficient diet for at least 21 weeks lost viability rapidly and had an increased production of thiobarbituric acid-reactive substances. This implies that severe vitamin E deficiency makes the cell more susceptible to oxidative stresses and that other oxidant defenses are unable to prevent lipid peroxidation under these conditions. These studies show that selenium-deficient hepatocytes and vitamin E-deficient hepatocytes can be isolated without special precautions. Thus, isolated hepatocytes can be used to study the effects of these nutrient deficiencies on xenobiotic metabolism and hepatotoxicity.

Selenium deficiency has a striking effect on glutathione turnover in the hepatocyte while the effect of vitamin E deficiency appears to be minimal. All cells are capable of synthesizing glutathione. Glutathione breakdown is catalyzed by γ-glutamylcysteine synthetase, which is virtually absent from rat hepatocytes. Therefore, turnover of glutathione in the hepatocyte would have to be ascribed to conjugation with a xenobiotic or ascribed to release from the cell. We have not added xenobiotics in these experiments.

Sies et al. (23) have studied glutathione release from the perfused liver and have reported that GSSG is released into the bile and GSH is released into the caval perfusate. Others have reported finding GSH as well as GSSG in the bile (16). Biliary GSSG release is increased by infusing hydroperoxides and certain xenobiotics into the liver (23). It is thought to occur as a result of redox changes in the liver leading to increased GSSG formation. GSH release into bile or into caval perfusate is unaffected under these conditions and factors which influence it have not been identified. Thus, previous studies provide no explanation of our observation that GSH release into the caval perfusate is increased by selenium deficiency.

Our studies provide evidence for accelerated glutathione synthesis in selenium-deficient hepatocytes when compared with controls. The activity of γ-glutamylcysteine synthetase is twice as high in selenium-deficient liver as in control. This enzyme catalyzes the initial step of glutathione synthesis and is considered, along with cysteine availability and other factors, to be involved in the regulation of glutathione synthesis (24-26). The glutathione content of the flask containing selenium-deficient hepatocytes increased by 105 nmol/10⁶ cells during the 5-h incubation while that of controls increased by only 45 nmol/10⁶ cells. Since little glutathione conjugation or degradation is likely to have occurred, this is strong evidence for increased synthesis in the selenium-deficient cells.

The evidence for increased glutathione synthesis in selenium deficiency is accompanied by evidence for an increased rate of GSH release into the blood. The perfused liver experiments (Table III) were necessary to determine that the augmented release of glutathione in selenium deficiency was into the blood instead of into the bile and that it consisted of GSH and not GSSG. An additional conclusion to be drawn from the perfusion experiments is that the increased GSH release is not related to artificially raising the glutathione synthesis rate by providing an excess of cysteine as could be argued was the case in the isolated hepatocyte experiments. The finding of 2 times as much glutathione in plasma from selenium-deficient rats as in controls (Table IV) supports the in vitro evidence of glutathione release.

Earlier studies have indicated that selenium deficiency has little or no effect on liver glutathione concentration (4). Therefore, the present data allow us to hypothesize that in vivo the increased release of GSH into the blood caused by selenium deficiency is balanced by an increased rate of glutathione synthesis in the hepatocyte so that intracellular glutathione concentration is maintained. The net result of this is an increase in plasma glutathione concentration (presumably GSH) and a decrease in cysteine concentration in the hepatocyte.

A major question raised by these findings is why the selenium-deficient liver releases much GSH into the blood. One possibility is that it leaks out due to membrane abnormality. We found no evidence of impaired viability of selenium-deficient cells in comparison to controls which might be expected if their membranes were abnormal, and they did not release lactic dehydrogenase into the perfusate. Thus, studies will be required to determine whether cell membranes are abnormal in selenium deficiency.

Another possibility is that GSH has an extracellular function and that the increased plasma concentration seen in selenium deficiency is a compensatory mechanism. Glutathione peroxidase and another selenoprotein are present in plasma (27), and declining concentrations of them might be compensated for by increased GSH. Future studies will have to determine whether extracellular GSH has an essential biochemical function.

The results of this study may shed some light on other phenomena observed in selenium deficiency. The accelerated glutathione synthesis could explain the protection against acetaminophen hepatotoxicity afforded by selenium deficiency in the rat (4). GSH is conjugated with a toxic metabolite of acetaminophen, thus detoxifying it. Its greater availability in selenium deficiency could allow detoxification of a greater quantity of the metabolite.
GSH Metabolism in Selenium-deficient Rat Hepatocytes

Selenium-deficient rats are more sensitive to inorganic mercury poisoning than are control rats. Several years ago, this finding was linked with lower levels of the protective mercury-binding protein metallothionein in rat kidney (28). One-third of the amino acid residues in metallothionein are cysteine. Thus, the low cysteine concentration seen in selenium deficiency could impair the induction of metallothionein and explain the sensitizing effect of selenium deficiency in inorganic mercury toxicity.

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