Dietary Magnesium Depletion: *p*-Nitroanisole Metabolism and Glucuronidation in Rat Hepatocytes and Hepatic Microsomal Membranes (43229)

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Abstract. **Male Sprague-Dawley rats were pair fed either a control magnesium diet (480 mg/kg) or a magnesium-deficient diet (30 mg/kg) for 10 days to produce magnesium depletion. Serum magnesium concentration declined 60%, but tissue magnesium deficiency was not apparent. Hepatic parenchymal cells and microsomal membranes were isolated to evaluate *in vitro* metabolism of *p*-nitroanisole and conjugation of its metabolite, *p*-nitrophenol (pNP). *O*-Demethylation of *p*-nitroanisole was decreased slightly in both magnesium depletion hepatocytes and microsomal membranes. However, pNP glucuronide formation was specifically decreased 50% in the magnesium depletion cells at all *p*-nitroanisole concentrations. Similar results were found in the microsomal reaction system measuring pNP conjugation. Thus, early stages of magnesium depletion may result in a specific decrease in the pNP UDP-glucuronyl transferase activity.**
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Altered nutritional status can impact drug and carcinogen metabolism, conjugation, and clearance (1). Starvation and protein, vitamin, and mineral deficiencies have all affected the hepatic system, primarily causing decreases in the mixed function oxidase components, cytochrome P-450 and cytochrome P-450 reductase, and drug metabolism (2, 3). However, little information exists for the early stages of nutrient depletion, which may more closely reflect the physiologic situation of the healthy, and medically compromised, population.

Isolated hepatocytes have served as important models of intact cellular metabolism (4–6), and have proven to provide great insight into the regulation of drug metabolism and conjugation (7–9), as well as providing an evaluation of cellular mechanisms of toxicity (10–12). Previously, Becking and co-workers (13, 14) have reported a decrease in drug metabolism and the mixed function oxygenase components following 12 days of dietary magnesium deficiency.

The experiments presented in this article were designed to evaluate the effect of magnesium depletion (10 days) on the metabolism of *p*-nitroanisole (pNA) by the mixed function oxidase and conjugation of its metabolite, *p*-nitrophenol (pNP), using both isolated hepatocytes and hepatic microsomes.

Materials and Methods

Animal Care and Feeding. Male Sprague-Dawley rats weighing 106 ± 5 g were fed a purified diet either adequate (480 mg/kg) or deficient in magnesium (30 mg/kg) and allowed to drink distilled water *ad libitum* (Table I). Control animals were pair-fed the same level of diet as consumed by the experimental group to counter changes produced by altered diet consumption. All animals were held in stainless steel cages and maintained in a room regulated for constant temperature (23°C), humidity (40%), and a 12-hr light:dark cycle. All animals were fed until the time of sacrifice.

Determination of Magnesium Status Using Serum and Liver Analysis. After intraperitoneal injec-

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Table I. Composition of Diet

Diet components	Amount
Casein (Sheffield Products, Norwich, NY)	20
Cornstarch (Dyets, Inc., Bethlehem, PA)	46
Sucrose (Sweeten, Vernon, CA)	20
Corn oil (Mazola)	5
Cellulose (ICN Nutritional Biochemicals, Cleveland, OH)	1
Vitamin mix[a]	2
Salt mix[b]	6
Total	100

[a] The composition of the vitamin mix (g/kg) was: thiamine, 0.6; riboflavin, 0.6; pyridoxine, 0.7; niacin, 3.0; pantothenate, 1.6; folic acid, 0.2; biotin, 0.02; cyanocobalamin, 0.001; vitamin A, 200,000 IU; vitamin E, 5,000 IU; vitamin D, 1250 IU; vitamin K, 0.005; sucrose, 972.9.

[b] The composition of the mineral mix (g/kg) was: calcium phosphate, 250; sodium chloride, 37; potassium sulfate, 26; potassium citrate, 110; magnesium carbonate (in control diet only), 30; manganous carbonate, 1.75; zinc carbonate, 0.8; cupric carbonate, 0.2; chromium acetate, 0.125; cobalt chloride, 0.015; sodium fluoride, 0.031; choline bitartrate, 57; potassium iodate, 0.005; ferrous gluconate, 4; sucrose, 513 (in the MgD mix) and 483 (in the control mix).

Table II. Differential Acid Hydrolysis for the Determination of *p*-Nitrophenol Conjugates

p-Nitrophenol standards	No hydrolysis	Hydrolysis method	
		7% trichloroacetic acid	12.5% HCl
pNP	100 ± 5.1[a]	100 ± 4.1	100 ± 7.1
pNP sulfate[b]	0	101 ± 5.8	101 ± 8.6
pNP glucuronide[b]	0	4.3 ± 4.3	96 ± 6.7

[a] The values are reported as mean percentage ± SD; 12–16 samples were evaluated for each parameter. pNP was used as the standard in each assay.

[b] Contained less than 2% free *p*-nitrophenol, and were so corrected.

Table III. Magnesium Status of 10-Day Magnesium-Depleted and Pair-Fed Control Sprague-Dawley Rats ($n = 6$)

	Magnesium concentration	
	Serum (μg/ml)	Liver (mg/g)
Control	23.6 ± 3.1	0.67 ± 0.04
Magnesium depleted	7.8 ± 1.8	0.72 ± 0.06
	$P < 0.001$	NS

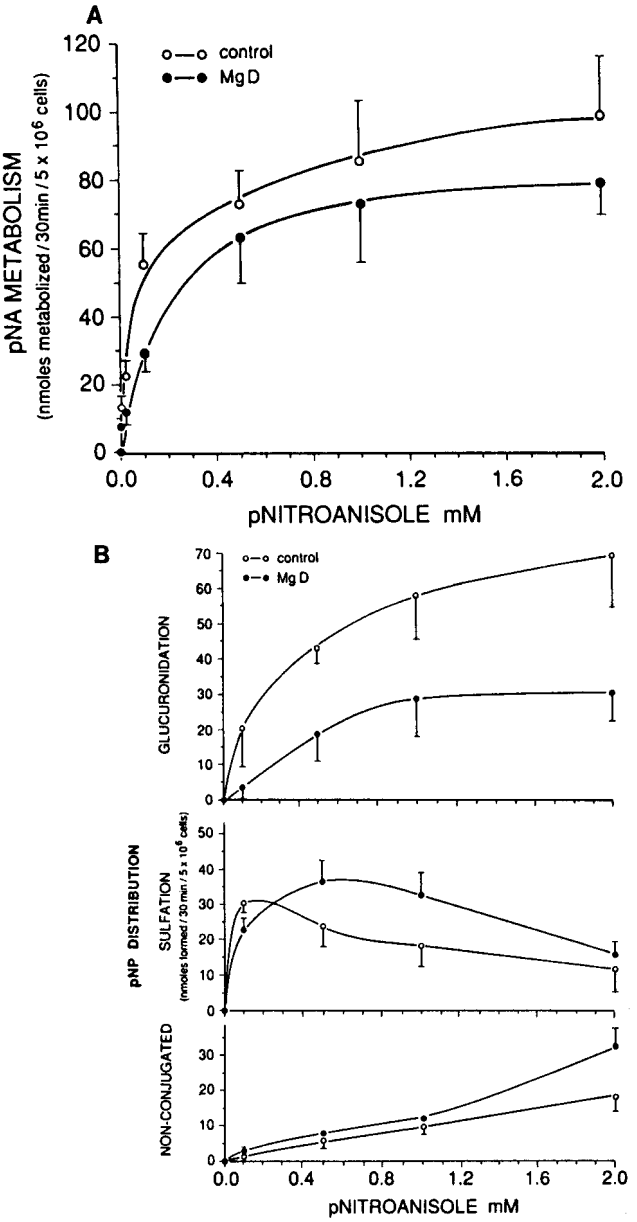

Figure 1. (A) Effect of magnesium depletion on *p*-nitroanisole *O*-demethylation in hepatocytes isolated from control and magnesium-depleted Sprague Dawley rats. The reaction conditions are described in Materials and Methods. Metabolism of pNA was evaluated from 0.0 to 2.0 m*M* in control and MgD hepatocytes. The rate of metabolism is expressed as nmol pNP formed/30 min/5 × 10^6 cells. No significant difference ($P < 0.05$) in metabolism was observed ($n = 5$). (B) Effect of magnesium depletion on *p*-nitrophenol conjugation during *p*-nitroanisole metabolism in isolated hepatocytes. Conjugation of pNP was evaluated at each pNA concentration. The rate of sulfation and glucuronidation during pNA metabolism is expressed as nmol/30 min/5 × 10^6 cells ($n = 5$).

tion of pentobarbital (45 mg/kg body wt), blood was withdrawn by cardiac puncture from control and magnesium-depleted animals. The collected blood was allowed to coagulate and the clot was removed by centrifugation at 1000 rpm in a table top clinical centrifuge. Serum magnesium was analyzed directly using a Perkin Elmer Atomic Absorption Spectrophotometer (model 703).

A liver sample (0.5 g) was weighed, dried for 24 hr at 110°C, and cooled in a desiccator, and the dry weight was recorded. Five milliliters of concentrated nitric acid

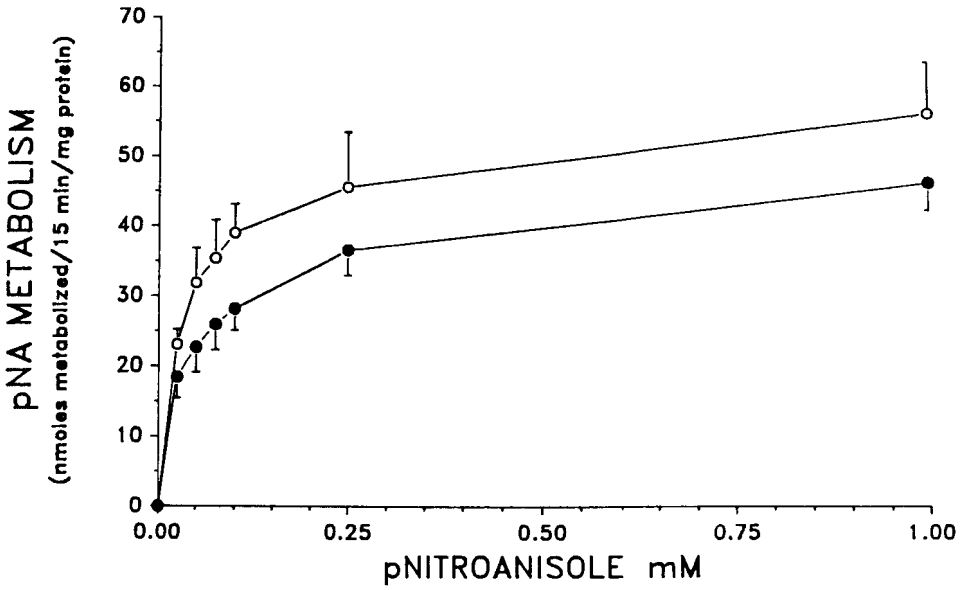

Figure 2. Effect of magnesium depletion on *p*-nitroanisole *O*-demethylation in hepatic microsomes isolated from control and magnesium-depleted Sprague-Dawley rats. The reaction conditions are described in Materials and Methods. The rate of metabolism is expressed as nmol of pNP formed/mg/15 min. The results were significantly different ($P < 0.05$) at all concentrations tested determined by the paired Student's *t* test ($n = 4$).

were added to the dried sample and the mixture was heated to digest the tissue. After most of the acid was evaporated, 30% H_2O_2 was added in 5-ml aliquots until the solution became clear. The sample was taken to dryness. The residue was redissolved in 0.3 *N* HCl and diluted to 5 ml in a volumetric flask. The magnesium was then quantified using a Perkin Elmer Atomic Absorption Spectrophotometer (model 703).

Hepatocyte Preparation. Hepatic parenchymal cells were prepared from liver by collagenase perfusion (4) and purified by a self-generating Percoll gradient (7). In brief, the liver was cannulated, flushed with calcium-free Hanks' bicarbonate buffer (pH 7.4), transferred to a perfusion chamber, and perfused further with Hanks' bicarbonate buffer, containing calcium chloride (14 mg/100 ml), collagenase (Type II, 15 mg/100 ml; Worthington), and trypsin inhibitor (Type I-5, 2 mg/100 ml; Sigma), which was recirculated for 25 min. All buffers were continuously bubbled with a mixture of 95% oxygen and 5% carbon dioxide. Removal of the Gillisons's capsule and gentle shaking provided free cells.

The concentration of cells in the hepatocyte suspension was determined and diluted to 5×10^6 cells/ml in Percoll having a final concentration of 31% (7). The Percoll-hepatocyte mixture was centrifuged under refrigeration for 10 min at 10,000*g*. The viable parenchymal cells formed a loose layer at the bottom of the Percoll gradient. Following removal of the excess Percoll and cellular debris by aspiration, the parenchymal cell layer was diluted 6-fold in Krebs-Henseleit bicarbonate (KHB) buffer (pH 7.4), and centrifuged at 2000*g* for 10 sec to wash the Percoll from the cells. The wash was repeated a second time.

Cell viability was assessed using the trypan blue exclusion method. Cells were diluted to about 10^6 cells/ml in isotonic saline containing 0.2% trypan blue. All hepatocytes used in these experiments had viability in excess of 90%.

Hepatocyte Metabolism of *p*-Nitroanisole. The 2.0-ml reaction system contained 5×10^6 cells and pNA (0–2 m*M*) in KHB buffer (pH 7.4). The reaction was carried out in 25-ml Erlenmeyer flasks containing an atmosphere of O_2:CO_2 (95:5). The temperature was maintained at 37°C in a shaking water bath. The reaction was initiated by addition of the cells. After 30 min, the reactions were terminated with 1.0 ml of ice-cold 20% trichloroacetic acid. The cell precipitate was removed by centrifugation at 2000*g* for 20 min. From the protein-free supernatant, 0.75-ml aliquots were assayed for the respective *p*-nitrophenol metabolites (Table II). (i) Free (nonconjugated) pNP was determined by the addition of 0.5 ml of 10% Na_2CO_3, adjusting the final pH to 9.5. Using a reference standard, the concentration of pNP was determined at 401 nm (15). (ii) *p*-Nitrophenolsulfate (pNPS) was determined by incubation of a separate aliquot at 100°C for 12 min to hydrolyze the sulfate bond. Under these conditions, no significant hydrolysis of the glucuronide bond was observed. Analysis of total pNP was carried out as described in (i). The difference between the total pNP and that initially present before hydrolysis provides the amount of pNPS formed. (iii) pNA metabolism was determined by complete hydrolysis of the conjugates to yield the total pNP formed. Addition of 0.75 ml of 25%

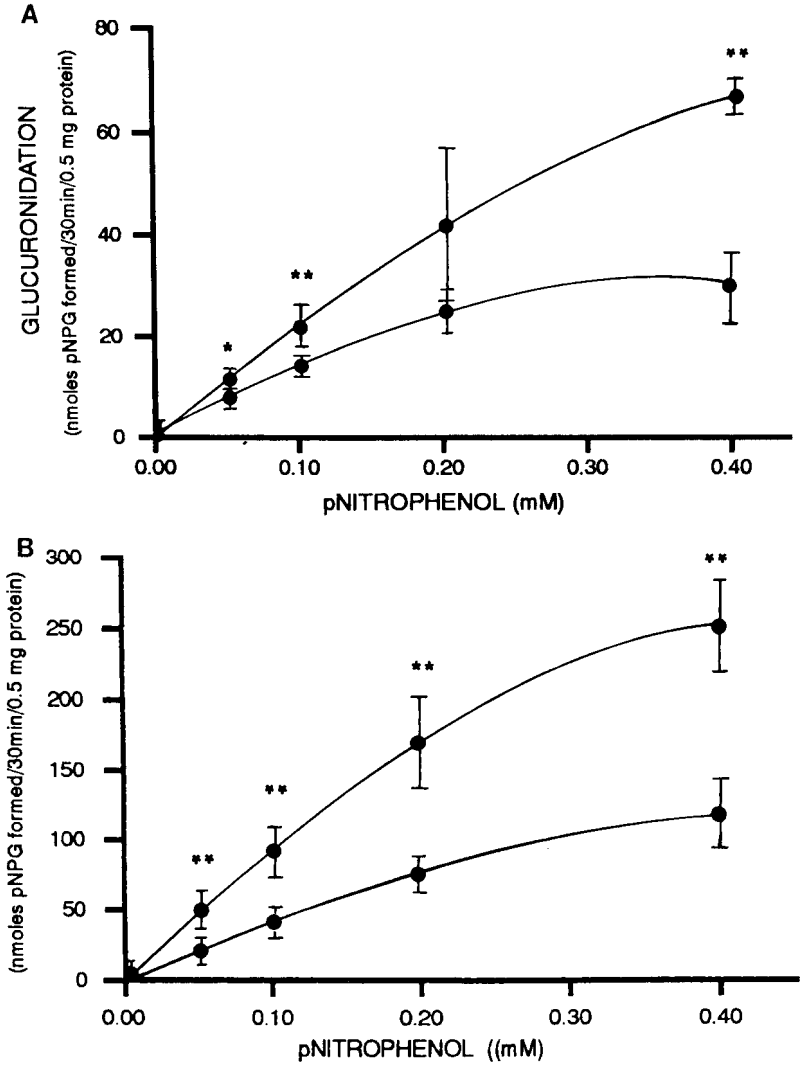

Figure 3. Effect of magnesium depletion on microsomal *p*-nitrophenol glucuronyl transferase activity. The reaction conditions are described in Methods. Conjugation of *p*-nitrophenol was evaluated from 0.0 to 0.4 m*M*, using control and MgD microsomes. The activity of glucuronyl transferase was determined as nmol of *p*-nitrophenol conjugated/30 min. The differences between control and MgD GT activities were significant (**P* < 0.05; ***P* < 0.01) as determined by the paired Student's *t* test. (A) Native GT activity. (B) Detergent-activated GT activity.

HCl to another aliquot and heating the mixture at 100°C for 60 min hydrolyzed both the sulfate and glucuronide bonds. Following hydrolysis, the pH of the reaction was adjusted to pH 9.5 by addition of 0.7 ml of 10 *N* NaOH, and the total pNP was determined at 401 nm. (iv) *p*-Nitrophenol glucuronide (pNPG) was determined by subtraction of the nonconjugated pNP and the amount of pNPS from the total pNP present.

Microsomal Preparation. The microsomal membrane was prepared by our modification of the method reported by Ernster *et al.* (16), as reported previously (17). The washed microsomal membranes were gently resuspended in a minimum volume of Tris (50 m*M*)-KCl (150 m*M*) buffer (pH 7.4) with a Teflon pestle. Protein was determined according to the method of Lowry *et al.* (18). The typical microsomal yield was 8–10 mg of microsomal protein/g of liver. The microsomal suspension was adjusted to 10 mg/ml.

Microsomal pNA Metabolism. The drug reaction system contained microsomes (1.0 mg/ml) suspended in 1 ml of Tris (50 m*M*)-KCl (150 m*M*) buffer (pH 7.4), containing $MgCl_2$ (5.0 m*M*), pNA (0.0–1.0 m*M*), and NADPH (0.5 m*M*). The reactions were carried out at 37°C for 15 min. All reactions were linear under these conditions.

The reactions were terminated by addition of ice-cold trichloroacetic acid (7% final). Following centrifugation at 2000*g* for 10 min, aliquots were removed from the clear supernatant fraction for pNP analysis. The demethylation of pNA was determined by the rate of formation of pNP, as described by Kato and Gillette (15). The amount of pNP formed in 15 min was meas-

ured at 401 nm and compared with a known pNP standard.

Determination of pNP Glucuronyl Transferase Activity. The native activity (19) of glucuronyl transferase (GT) was measured in the liver microsomal fraction using 0 to 0.4 m*M* pNP, 2.5 m*M* UDP-glucuronic acid (UDPGA), 0.5 mg microsomal protein in a KHB buffer (pH 7.4) with or without $MgCl_2$ (5 m*M* final), as described previously (17). The reactants were preincubated for 10 min at 37°C. The reaction was initiated by the addition of UDPGA. To determine total GT activity, the microsomes were activated by the addition of Triton X-100 (0.1% final concentration) and preincubated at room temperature for 10 min (20). Activity was reported as nmol of pNP conjugated in 30 min/0.5 mg of microsomal protein.

Statistical Methods. The statistical significance of the results was tested by the paired Student's *t* test. All values are reported as the mean ± SD (21).

Results

Magnesium depletion was produced in male Sprague-Dawley rats by feeding a magnesium-deficient (MgD) diet (30 mg/kg), while normal magnesium status was maintained by feeding a pair-fed control (PFC) diet (480 mg/kg), for 10 days. Both groups of animals continued to gain weight (39 ± 8 g) and maintained the appearance of *ad libitum*-fed controls. A major decrease (67%) in plasma magnesium concentration was observed between the PFC (23.6 ± 3.1 μg/ml) and the MgD animals (7.8 ± 1.8 μg/ml), yet no significant change in the magnesium concentration of liver tissue was observed (Table III).

Metabolism of pNA was evaluated in hepatocytes isolated from MgD and pair-fed control animals. Use of intact cells allowed evaluation of the effect of magnesium depletion on drug metabolism and conjugation over a pNA concentration range of 0.0–2.0 m*M*. The primary product of pNA *O*-demethylation is pNP. A consistent, but nonsignificant, decrease in pNA metabolism was observed at all concentrations in MgD cells compared with the control hepatocytes (Fig. 1A). This result closely parallels the small decrease observed in microsomal metabolism of pNA in MgD microsomes relative to PFC microsomes (Fig. 2).

Within the cell, the resulting product, pNP, is metabolized further by sulfotransferase and GT (Fig. 1B). In the PFC cells, at 0.1 m*M* pNA, the mixed function oxygenase reaction produced about 55 nmol of pNP during the reaction period. At this point, sulfate conjugation (30 nmol) exceeded glucuronidation (20 nmol). At higher pNA concentrations, the production of pNP doubled; however, sulfation decreased about 50%, whereas glucuronidation increased 3.5 times.

In the MgD cells, sulfate conjugation was not limited by the depletion of magnesium, indicating that the synthesis of adenosine 3′-phosphate, 5′-phosphosulfate did not differ from the PFC cells and sulfotransferase was not limited for cellular conjugation. At 0.1 m*M* pNA, the production of pNPS was slightly lower than in the PFC cells, but at all higher concentrations of pNA the MgD cells produced more pNPS than the PFC cells.

As the concentration of pNA increased, the PFC cells produced a greater amount of pNP glucuronide. Above 0.1 m*M* pNA, and greater percentage of pNPG was formed relative to pNPS. Glucuronidation also increased in the MgD cells, but the total pNPG formed was less than half of the glucuronide synthesized in the PFC cells. Since the total amount of pNP was similar in both cells, the decrease in glucuronidation in the MgD cells could have resulted from a specific loss of the GT isoenzyme.

To evaluate this possibility, the activity of GT was examined further in hepatic microsomes prepared from PFC and MgD animals (Fig. 3). The native and detergent (Triton X-100)-treated activity was determined in the presence of 5 m*M* $MgCl_2$. The rate of glucuronidation in the MgD animals was half that of the control animals. These effects were noted in both the native and detergent-treated enzyme activities. Thus, the results indicated that the GT isoenzyme activity was specifically lost during Mg depletion.

The observed increase in pNPS in the MgD cells may well result from the less efficient rate of glucuronidation. This interpretation is also supported by the increase in nonconjugated pNP present in the cells at the highest pNA concentrations.

Discussion

Almost 20 years ago, the effect of magnesium deficiency on microsomal drug metabolism was reported. Becking and Morrison (13) and Becking (14) reported a decrease in cytochrome P-450 by Day 11 and a reduction in NADPH cytochrome *c* reductase activity at 12 days of dietary depletion of magnesium (5 mg/100 g diet). A corresponding loss of drug metabolism also occurred. More recently, Bidlack and Brown (22) reported a decrease of cytochrome P-450 and a decrease in pNA and aniline metabolism by 18 days; however, these animals had physical characteristics of Mg deficiency and appeared undernourished.

In the current studies, the metabolic changes that occur during Mg depletion (early stages of magnesium deficiency) were examined. Dietary restriction of Mg for 10 days resulted in a slight decrease in pNA metabolism in both isolated hepatocytes and isolated microsomal membranes. The intact cell system allowed evaluation of the conjugation reactions as well.

Sulfotransferase and glucuronyl transferase can simultaneously metabolize the same substrate, and at the same time compete for those substrates (7, 10). The

level of pNP available for conjugation was similar in both the MgD and the PFC cells. Since the level of pNPS formed was as much as 30% greater than in the PFC cells, sulfotransferase activity did not appear to be limited in the MgD cells. Conversely, the formation of pNPG was decreased more than 50% in the MgD cells.

Although the decrease in glucuronide formed could have resulted from the unavailability of UDPGA, a lack of cation regulation or a lack of the enzyme, the decrease in glucuronidation was concluded to result from a specific loss of the glucuronyl transferase activity. Using hepatic microsomes from control and MgD animals, the MgD pNP-GT activity remained low even in the presence of $MgCl_2$ and saturating concentrations of UDPGA; similar results were obtained from native and detergent-treated GT activity. These results suggest that a very subtle change in Mg status has produced a specific decrease in the pNP-GT isoenzyme.

Multiple isoenzyme forms for GT have been identified by chromatographic separation, substrate specificity, and quantification of kinetic parameters (23–26). Hochman and Zakim (24) isolated two pNP-GT isoenzymes from pig liver, having two isoenzymes with a 100-fold difference in V_{max}. Only the low V_{max} enzyme was activated by addition of divalent cations. From rat liver, Chowdhury *et al.* (25) have isolated three pNP-GT isoenzymes, but the authors did not evaluate individual isoenzyme sensitivity to magnesium.

The different isoenzyme forms of GT are under different genetic regulatory control, as indicated by induction with 3-methylcholanthrene and phenobarbital (19, 23, 26). However, the evaluation of genetic expression of related isoenzymes, e.g., multiple pNP-GT isoenzymes, has not been reported.

The most intriguing aspect of the *in vitro* results reported here is that without a significant change in total liver magnesium, the pNP-GT isoenzyme was specifically decreased. The mechanism by which Mg depletion produced the reduction of the pNP-GT is currently under investigation.

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