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EFFECTS OF PROPYLTHIOURACIL ON D-GALACTOSAMINE HEPATOTOXICITY IN THE RAT

EVIDENCE FOR A NON-THYROIDAL EFFECT*

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Abstract—The cytoprotective effects of propylthiouracil (PTU) were studied in rats treated with the hepatotoxin D-galactosamine (D-GNH_2). Five days of PTU pretreatment prior to D-GNH_2 caused hypothyroidism and a significant reduction in liver injury as assessed by serum transaminase levels. When PTU was administered as a single dose with D-GNH_2, significant decreases in transaminase also occurred at times when thyroid function was unchanged. Furthermore, aminopyrine oxidation showed significant impairment after D-GNH_2 and was normalized by one dose of PTU. Further studies were carried out in thyroidectomized rats. PTU caused significant reductions in transaminase levels when given for 5 days pretreatment or as a single dose. Animals receiving pretreatment with PTU plus thyroxine (T_4) also had significant decreases in serum transaminase. The antithyroid drug methimazole also had a hepatoprotective effect, while two other potent antithyroid compounds (2-thiouracil and 2-thiobarbituric acid) did not. These data suggest that PTU can protect against liver injury induced by D-GNH_2, that the effect is independent of thyroid function, and that this effect is not common to all thiol-containing antithyroid drugs.

The antithyroid drug propylthiouracil (PTU) has been shown to protect against the development of toxic liver injury caused by ethanol [1, 2], carbon tetrachloride [3], and acetaminophen [4–6]. Because the hyperthyroid state increases and the hypothyroid state decreases the severity of hepatocellular dysfunction in several experimental systems [7–14], it has been postulated that the cytoprotective effects of PTU are secondary to hypothyroidism induced by this antithyroid drug [1, 2, 4, 15]. Recently, however, it has also been suggested that the antihepatotoxic activity of PTU may be independent of its effect on the thyroid [5]. In studies of acetaminophen toxicity, for example, Yamada *et al.* [5] demonstrated that PTU forms nontoxic complexes with potentially toxic acetaminophen metabolites, thereby preventing covalent binding of acetaminophen to liver macromolecules. Studies in humans have suggested a possible beneficial effect of PTU in alcoholic liver disease [16], but more recent observations have not confirmed the protective effects of PTU either in alcoholic hepatitis in man [17] or in experimental murine hepatitis [18].

The present studies were conducted to further define the actions of PTU in experimental liver injury and to examine in detail the relationship between its capacity to protect the liver and its antithyroid activity. In addition, structure–activity studies of various PTU analogues were performed to define which parts of the PTU molecule were essential for a protective effect. D-Galactosamine (DGNH_2), a potent and selective hepatotoxin [19–21], was utilized in these studies as an agent known to cause severe, but reversible, hepatic damage.

MATERIALS AND METHODS

Reagents

L-Thyroxine, D-GNH_2, 1-methyl-2-mercaptoimidazole (methimazole), 2-thiobarbituric acid (2-thio-4,6-dihydroxypyrimidine), 2-thio-6-propyluracil (PTU), and 2-thiouracil were obtained from the Sigma Chemical Co. (St. Louis, MO). Propyluracil and *S*-methyl-6-propylthiouracil were synthesized according to the method of Lindsay *et al.* [22]. All antithyroid drugs and derivatives were dissolved in 1 N NaOH, and the pH was adjusted to 9.0 with 1 N HCl. 4-Dimethyl[^{14}C]aminopyrine (sp. act. = 114 mCi/mmole) was obtained from the Amersham Corp., Arlington Heights, IL.

Animals

Intact and surgically thyroidectomized female Wistar rats weighing 180–200 g were purchased from the Charles River Breeding Laboratories (Wilmington, MA). Conditions of animal maintenance were similar to those reported previously [23]. Tap water and a standard rat food (Purina rodent laboratory chow No. 5001) were provided *ad lib.* Animals were fasted overnight prior to the administration of D-GNH_2. Serum and hepatic tissue were obtained after anesthesia with ether and exsanguination via the aorta.

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Experimental studies

Experiment I. This study was performed to assess whether PTU was capable of modifying D-GNH_2-induced hepatic injury, using standard PTU (50 mg/kg, i.p.) [1, 3], and D-GNH_2 (500 mg/kg, i.p.) doses. Rats were divided into the following groups containing fifteen to thirty animals each: Group 1, no treatment; Group 2, treatment with PTU (50 mg/kg, i.p.) daily for 10 days; Group 3, D-GNH_2 (500 mg/kg, i.p.) as a single dose on day 0; Group 4, pretreatment with PTU daily for 5 days (days −4 to 0), followed by a single dose of D-GNH_2 on day 0, with daily PTU until sacrifice. Five days of PTU has been shown previously to be a sufficient time to induce hypothyroidism [24]. Group 5, single dose of PTU plus a single dose of D-GNH_2 on day 0 (administered 1 min apart); and Group 6, D-GNH_2 on day 0, followed by single doses of PTU 4, 8, or 12 hr after D-GNH_2.

Twenty-four, 48, 72 and 96 hr after D-GNH_2, six to eight animals from Groups 1 through 5 were killed. Group 6 animals were autopsied at only one time, 24 hr after D-GNH_2. One hour prior to autopsy, animals were injected with 20 μCi [^{3}H]thymidine (sp. act. 6 μCi/mmole, New England Nuclear Corp.) via tail vein for subsequent [^{3}H]DNA analysis as an index of hepatic repair. The functional status of the livers from three to four additional animals in Groups 1, 3 and 5 was assessed by measuring aminopyrine oxidation. Twenty-four hours after D-GNH_2 (Group 3) or D-GNH_2 plus PTU (Group 5), 0.25 μCi of labeled aminopyrine and 9 mg/kg of carrier aminopyrine were injected i.p. Immediately following the injection, each rat was placed in a breath collection chamber, and the $^{14}CO_2$ was collected as previously described [25].

Hepatic histology was assessed after 10 days in Group 2 animals and 24 hr after D-GNH_2 in animals in Groups 3 and 5. Portions of liver were removed, fixed immediately in 10% buffered formalin, and submitted for histologic analysis to Metpath Laboratories (Teterboro, NJ). Sections were embedded in paraffin, stained with hematoxylin and eosin, and analyzed in a blinded manner by a certified veterinary pathologist.

Experiment II. This experiment was performed to investigate whether PTU would protect against D-GNH_2-induced liver injury in rats made hypothyroid independent of PTU treatment, and in hypothyroid rats given replacement doses of L-thyroxine. PTU and D-GNH_2 doses were 50 mg/kg and 500 mg/kg, respectively, as in Experiment I. Two weeks after surgical thyroidectomy, rats were divided into the following groups (N = 28–32): Group 1, no treatment; Group 2, D-GNH_2 as a single dose on day 0; Group 3, pretreatment with PTU for 5 days (days −4 to 0), followed by a single dose of D-GNH_2 on day 0 with daily PTU until sacrifice; Group 4, single dose of PTU plus single dose of D-GNH_2 on day 0 (administered 1 min apart); and Group 5, pretreatment with PTU plus L-thyroxine, 10 μg/kg i.p., daily for 5 days (days −4 to 0), followed by D-GNH_2 on day 0.

Animals in Groups 1–3 were autopsied at 24, 48, 72, and 96 hr after D-GNH_2, following pretreatment with [^{3}H]thymidine as in Experiment I. Animals in Groups 4 and 5 were autopsied at 24 hr only.

Experiment III. This study was performed to assess structure–activity relationships of various PTU analogues. Rats were divided into the following groups: Group 1 (N = 50), D-GNH_2 alone (ten to twelve rats per experiment for five experiments); and Groups 2–6 (N = 14), D-GNH_2 plus one of the following: *S*-methyl-6-propylthiouracil, methimazole, propyluracil, 2-thiobarbituric acid, or 2-thiouracil administered 1 hr later. Doses of drugs were equimolar with 50 mg/kg of PTU (i.e. 0.29 mmole/kg).

Animals were autopsied 24 hr after D-GNH_2 administration.

Assays

Serum thyroxine (T_4) and thyrotropin (TSH) were used to assess thyroid function and were measured by previously described radioimmunassays [23, 26]. [^{3}H]Thymidine incorporation into liver DNA was measured by a previously described technique [27]. Serum glutamine oxalacetic transaminase (SGOT) was used to measure liver damage and was assayed spectrophotometrically using 10 μl of serum. Studies were performed in which PTU was added directly to sera with elevated SGOT (1000–2000 I.U.). No effect on SGOT was seen even at a PTU concentration of 250 μg/ml, which is 10- to 1000-fold higher than would be seen *in vivo* [28].

Statistics

Data were expressed as mean ± S.E. The linearity and correlation of data were assessed using the method of least squares. Multiple comparisons between the means of control and treatment groups were made by Tukey's wholly-significant-difference procedure [29].

RESULTS

Experiment I (Fig. 1)

Control SGOT values were 240 ± 20 I.U.; 10 days of PTU treatment had no effect on serum SGOT. A single dose of 500 mg/kg D-GNH_2 caused severe hepatocellular damage, with peak SGOT levels of

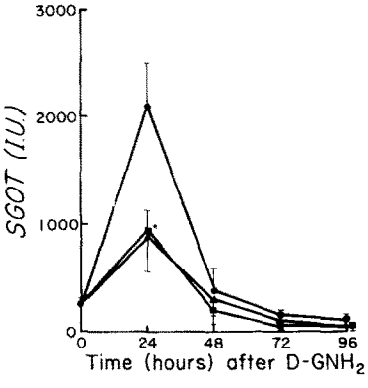

Fig. 1. SGOT levels after D-GNH_2 in intact animals receiving D-GNH_2 alone (●—●), D-GNH_2 after 5 days of PTU pretreatment (▲—▲), or D-GNH_2 and PTU simultaneously (■—■). Key: (*) $P < 0.02$ compared to D-GNH_2 alone. Seven to eight animals were used for each data point.

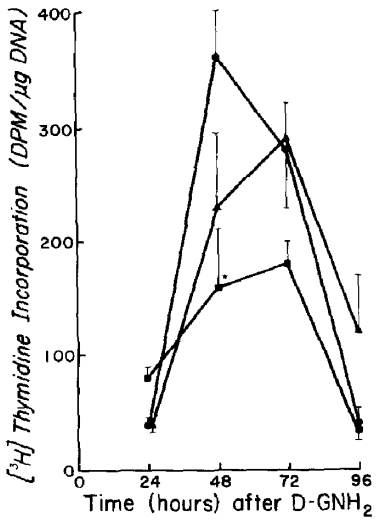

Fig. 2. [^{3}H]Thymidine incorporation into DNA in intact animals receiving D-GNH_2 (●—●), DGNH_2 after 5 days of PTU pretreatment (▲—▲), or D-GNH_2 and PTU simultaneously (■—■). Key: (*) $P < 0.02$ compared to D-GNH_2 alone. Seven to eight animals were used for each data point.

2037 ± 442 I.U. 24 hr after injection ($P < 0.001$ compared to control). In contrast, PTU pretreatment for 5 days prior to D-GNH_2 sharply reduced peak SGOT levels to 875 ± 172 I.U. ($P < 0.02$ compared to D-GNH_2 alone). A similar protective effect on hepatic injury was noted when PTU was administered simultaneously with D-GNH_2, with a peak SGOT value of 975 ± 393 I.U. ($P < 0.01$ vs D-GNH_2 alone). Furthermore, a protective effect was also observed in animals that received PTU, 4, 8, or 12 hr after D-GNH_2, with serum SGOT measured 24 hr after D-GNH_2 of 1128 ± 421, 408 ± 68, and 631 ± 66 I.U. respectively (P = NS for 4 hr, $P < 0.01$ for the 8- and 12-hr time points). Hepatic repair, assessed by [^{3}H] thymidine incorporation into DNA, followed the time course of hepatocellular injury (Fig. 2). At 48 hr, hepatic repair was significantly *less* in the groups receiving D-GNH_2 and PTU simultaneously compared to the group receiving D-GNH_2 alone (160 ± 48 vs 360 ± 80 dpm/μg DNA, $P < 0.02$). This is consistent with the diminished hepatic injury observed in the PTU-treated animals (Fig. 1).

Table 1. Effects of D-GNH_2 and PTU on [^{14}C]aminopyrine oxidation *in vivo*

Treatment	N*	Cumulative $^{14}CO_2$ radioactivity† (%)
None	4	16.8 ± 1.1
D-GNH_2‡	3	11.3 ± 2.8§
PTU	3	17.7 ± 1.1
D-GNH_2 + PTU	3	18.6 ± 1.8

* Number of animals used.
† $^{14}CO_2$ was measured 1 hr after [^{14}C]aminopyrine administration.
‡ D-GNH_2 was administered 24 hr prior to testing.
§ $P < 0.001$ compared to control.

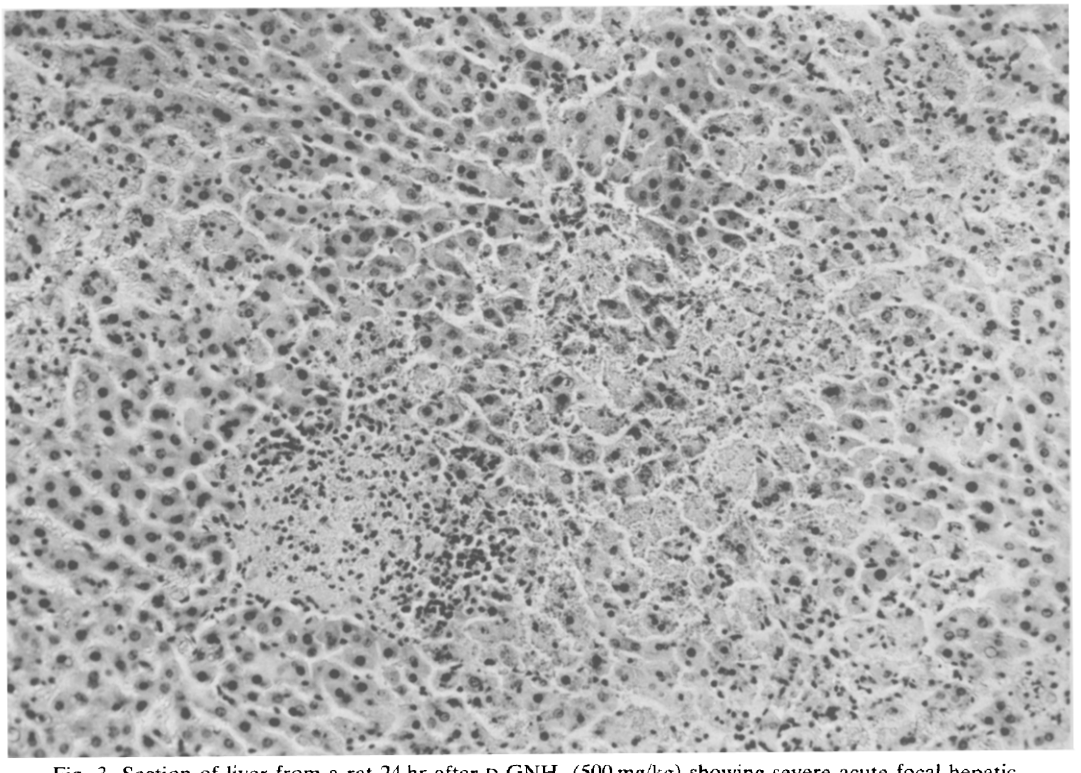

Fig. 3. Section of liver from a rat 24 hr after D-GNH_2 (500 mg/kg) showing severe acute focal hepatic necrosis (×150).

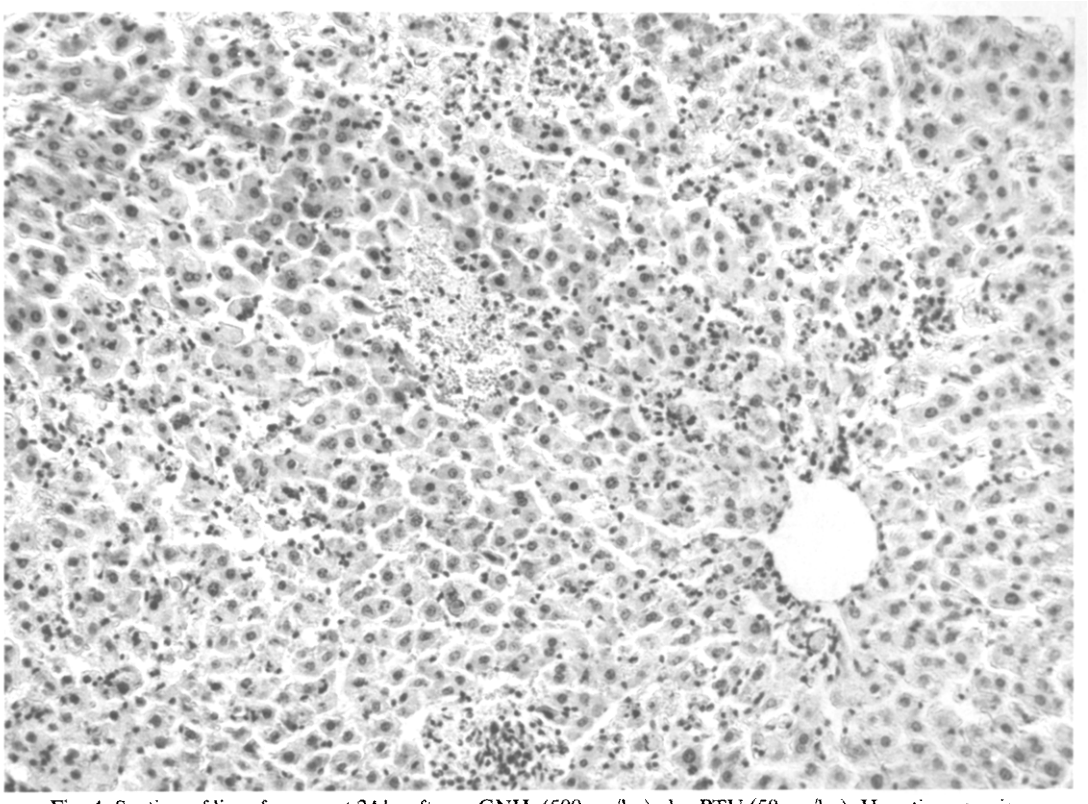

Fig. 4. Section of liver from a rat 24 hr after D-GNH_2 (500 mg/kg) plus PTU (50 mg/kg). Hepatic necrosis is less severe (×150).

The aminopyrine oxidation data are displayed in Table 1. Approximately 17% of the aminopyrine was oxidized to $^{14}CO_2$ in 1 hr by normal control rats (Group 1). This is similar to what we have observed previously in mice [25]. Pretreatment of the rats with D-GNH_2 (Group 3) reduced aminopyrine oxidation significantly ($P < 0.001$), while pretreatment with PTU had no effect. The inhibitory effect of D-GNH_2 on aminopyrine oxidation was prevented completely by the co-administration of PTU (Group 5).

Histologic examination was performed on livers from animals in Group 2 (PTU only), Group 3 (D-GNH_2 alone), and Group 5 (D-GNH_2 plus single-dose PTU). Animals exposed to D-GNH_2 alone had severe acute focal hepatic necrosis (Fig. 3), while animals given D-GNH_2 plus PTU had less severe damage (Fig. 4), consistent with the SGOT data. Sections of liver from animals given only PTU were normal (data not shown).

The effects of PTU on thyroid function are shown in Table 2. Control serum T_4 and TSH concentrations were 5.3 ± 0.3 μg/dl and 1.7 ± 0.6 ng/ml respectively. Values were unaffected by D-GNH_2 administration alone (Group 3). In Group 4 animals, pretreated with PTU for 5 days, T_4 levels were significantly lower and TSH was significantly higher at 48, 72, and 96 hr after D-GNH_2, indicating that PTU exerted an antithyroid effect within 7 days of treatment. Group 5 animals, which received only one dose of PTU, had a transient decrease in T_4 (1.9 ± 1.0 μg/dl, $P < 0.02$), without a change in TSH, only at 72 hr post-injection of PTU; by 96 hr post-injection, T_4 had returned to normal values (5.6 ± 0.7 μg/dl). Group 6 animals were killed only

Table 2. Serum thyroxine (T_4) and Thyrotropin (TSH) in rats treated with D-GNH_2 with and without PTU

	T_4 (μg/dl)				TSH (ng/ml)			
	24 hr	48 hr	72 hr	96 hr	24 hr	48 hr	72 hr	96 hr
Control	5.3 ± 0.3	4.6 ± 0.6	5.3 ± 1.0	5.0 ± 0.7	1.7 ± 0.6	0.9 ± 0.1	1.4 ± 0.2	1.3 ± 0.3
D-GNH_2	3.7 ± 1.5	3.1 ± 0.7	5.3 ± 2.3	6.1 ± 0.9	1.6 ± 0.5	1.0 ± 0.3	0.9 ± 0.2	1.3 ± 0.2
Daily PTU + D-GNH_2	2.6 ± 1.2	1.5 ± 0.5*	1.8 ± 1.0†	1.5 ± 0.7‡	3.6 ± 2.8	5.0 ± 1.9‡	6.1 ± 0.8‡	7.6 ± 1.5‡
PTU × 1 dose + D-GNH_2	6.0 ± 1.5	3.9 ± 0.7	1.9 ± 1.0†	5.6 ± 0.7	1.2 ± 0.6	1.9 ± 1.8	1.3 ± 0.4	1.3 ± 0.3

* $P < 0.01$ vs D-GNH_2 only.
† $P < 0.02$ vs D-GNH_2 only.
‡ $P < 0.001$ vs D-GNH_2 only.

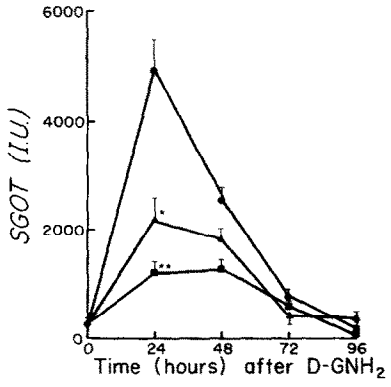

Fig. 5. SGOT levels after D-GNH_2 in thyroidectomized animals receiving D-GNH_2 alone (●—●), D-GNH_2 after 5 days of PTU pretreatment (▲—▲), or D-GNH_2 and PTU simultaneously (■—■). Key: (*) $P < 0.02$ and (**) $P < 0.01$ compared to D-GNH_2 alone. Seven to eight animals were used for each data point.

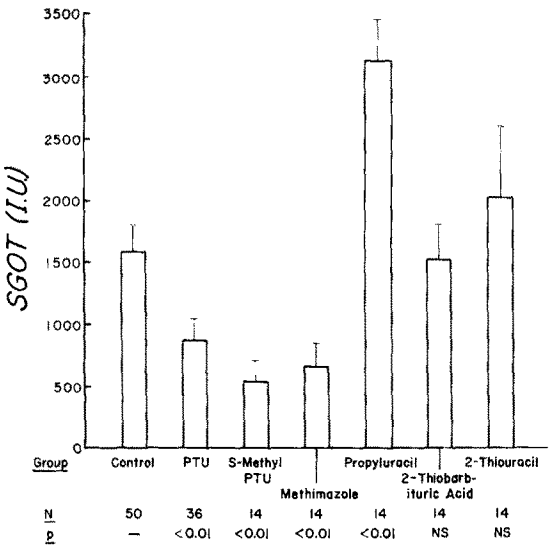

Fig. 7. Effects of PTU and various analogues (0.29 mmole/kg) on liver injury induced by D-GNH_2 (500 mg/kg).

at 24 hr post D-GNH_2. Thyroid function at the time of sacrifice was normal in all animals.

Experiment II (thyroidectomized rats)

Mean basal T_4 values for these thyroidectomized rats were 0.6 ± 0.1 μg/dl with TSH levels of 13.2 ± 0.6 ng/ml, reflecting their hypothyroid state. No further changes in thyroid function were noted with D-GNH_2 or D-GNH_2 plus PTU. In animals receiving T_4 plus PTU, serum T_4 was 6.2 ± 0.5 μg/dl with TSH levels of 4.0 ± 1.2 ng/ml, consistent with a return toward the euthyroid state with T_4 treatment.

Data for D-GNH_2 administration are shown in Fig. 5. Control animals had normal SGOT values (data not shown). After D-GNH_2 alone, peak SGOT levels were 4945 ± 536 I.U. at 24 hr. Significant decreases in SGOT were noted when animals were pretreated with PTU for 5 days (peak SGOT = 2247 ± 556 I.U., $P < 0.02$) or when D-GNH_2 and PTU were administered simultaneously (peak SGOT = 1040 ± 122 I.U., $P < 0.01$). The difference in peak SGOT levels between the two PTU treatment groups was not statistically significant. Animals receiving the combination of T_4 (10 μg/kg) and PTU prior to D-GNH_2 had peak SGOT levels of 1448 ± 201 I.U. ($P < 0.001$ compared to D-GNH_2 alone) (data not shown). There was no significant difference between SGOT levels in animals receiving T_4 and PTU compared to animals receiving PTU alone.

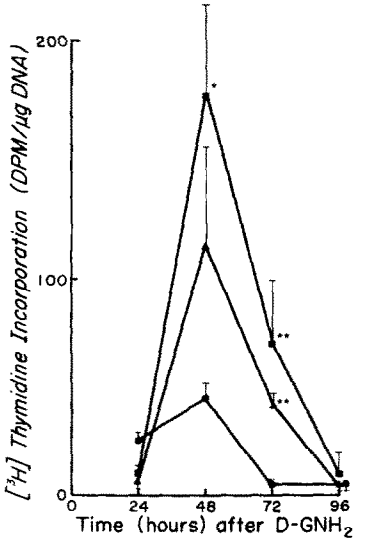

Fig. 6. [^{3}H]Thymidine incorporation into DNA in thyroidectomized animals receiving D-GNH_2 alone (●—●), D-GNH_2 after 5 days of PTU pretreatment (▲—▲), or D-GNH_2 and PTU simultaneously (■—■). Key: (*) $P < 0.05$ and (**) $P < 0.01$ compared to D-GNH_2 alone. Seven to eight animals were used for each data point.

In general, hepatic repair was less active in the thyroidectomized rats compared to the intact rats in Experiment I (Fig. 6). This is consistent with previous observations of hepatic regeneration in hypothyroidism [30]. In contrast to what was seen in intact animals, [^{3}H]thymidine incorporation into DNA was significantly higher in PTU-treated thyroidectomized animals at several time points after D-GNH_2, despite the fact that liver injury was less severe than in the thyroidectomized animals receiving D-GNH_2 only.

Experiment III (PTU analogue studies)

Studies with various PTU analogues are shown in Fig. 7. Mean SGOT levels 24 hr after D-GNH_2 were 1535 ± 215 I.U. Significant decreases in SGOT were observed with PTU (50 mg/kg) and equimolar doses of *S*-methyl PTU and methimazole. The PTU metabolite propyluracil actually significantly increased liver injury as assessed by SGOT, while the antithyroid compounds 2-thiouracil and 2-thiobarbituric acid had no effect on the severity of liver injury.

DISCUSSION

It has long been appreciated that the hyperthyroid state renders the liver more susceptible to toxic or metabolic injury. Studies with chloroform [7], anoxia

[8], choline deficiency [9, 31], carbon tetrachloride [10], and anesthetic agents [13, 14] all demonstrate a worsening of hepatic function when thyroid hormones are co-administered at the time of liver insult. Based on these data, it was hypothesized that the induction of hypothyroidism by an antithyroid drug might serve to protect the liver against injury. Indeed, a number of older reports demonstrated that therapy with goitrogenic compounds, such as thiouracil and aminothiazole, could modify several forms of diet-induced cirrhosis in rats [9, 31–33].

Recent studies have also shown that treatment with the antithyroid drug PTU leads to a significant reduction in hepatic injury induced by ethanol [1, 2], carbon tetrachloride [3] and acetaminophen [4–6]. The present study provides functional data which document that PTU is also effective in ameliorating the liver damage associated with D-GNH_2 exposure. This is of particular interest since, unlike ethanol, carbon tetrachloride, and acetaminophen, D-GNH_2 is possibly not a direct hepatoxin [19]. Recently, Mihas *et al.* have presented data showing that PTU pretreatment for 10 days actually increased hepatic damage after D-GNH_2, observations which are completely contrary to the results of the present study. Also, Nadkarni *et al.* [35] found no protection by PTU in a D-GNH_2 plus chronic ethanol rat model. At present, we have no explanation for these discrepancies with the present study.

The present studies demonstrate that PTU had a protective effect on the liver without inducing a hypothyroid state. While it is true that a protective effect of PTU was noted when animals were rendered severely hypothyroid after PTU treatment (i.e. Group 4, Experiment I), similar effects were seen even with single doses of PTU which caused little or no change in thyroid function (i.e. Groups 5 and 6, Experiment I). Furthermore, we noted that PTU was also active in preventing liver injury in severely hypothyroid animals, as well as in thyroidectomized animals replaced with exogenous thyroid hormone. These data strongly suggest that the ability of PTU to protect against D-GNH_2-induced liver damage was due to a direct hepatic effect. Our observations are consistent with the early studies of Borell and Holmgren [36], who showed that the antithyroid compound methylthiouracil decreased hepatic oxygen consumption significantly in thyroidectomized animals. Subsequent studies, demonstrating disparate effects of thyroidectomy and thiouracil treatment on hepatic protein metabolism [37], also pointed towards a nonthyroidal hepatic effect of thiouracil derivatives.

More recently, data consistent with this hypothesis have been obtained in several experimental models of liver injury. In the acetaminophen hepatotoxicity system, for example, PTU has been shown to render a protective effect by forming covalently-linked conjugates with reactive and potentially toxic acetaminophen metabolites [5]. Since PTU is a competitive inhibitor of GSH for GSH *S*-transferase [38], increased hepatocellular GSH concentrations may be an additional mechanism involved in the protective effects of PTU. Furthermore, in studies using ethanol, Nadkarni *et al.* [39] demonstrated that PTU reversed a generalized decrease in plasma protein synthesis induced by ethanol treatment, and Moreno *et al.* [40] have shown that PTU increased the activity of the hepatic microsomal ethanol-oxidizing system, independent of its thyrostatic effects. Finally, Cook *et al.* have recently shown *in vitro* that PTU protects against thyroid hormone augmented salicylate hepatotoxicity, although no effect on salicylate toxicity in the absence of thyroid hormone could be demonstrated [41].

The data in the present study do not provide a mechanism by which PTU protects against liver injury. This question is particularly difficult to answer in the D-GNH_2 model, since the underlying pathogenesis of D-GNH_2 hepatotoxicity is complex. There is a considerable amount of evidence to suggest that D-GNH_2 administration depletes cells of uridine nucleotides, with a concomitant decrease in RNA levels and protein synthesis [20]. *In vivo* and *in vitro* studies demonstrating the reversibility of D-GNH_2 toxicity by uridine are supportive of this view [20, 42–44]. On the other hand, recent work has focused on the possibility that D-GNH_2-mediated liver injury may be related to extrahepatic factors, especially the release of endotoxin from gut bacteria and activation of the complement system [21, 45].

Regardless of the mechanism of D-GNH_2 liver injury, the mitigating effects of PTU are not explained easily. It is unlikely that PTU directly inactivates D-GNH_2, since liver injury was preventable (or reversible) by PTU injected 12 hr after D-GNH_2 was administered. Previous studies have shown that hepatocellular changes occur within 2–4 hr of D-GNH_2 administration [46–48]. Although thiouracil is not directly incorporated into RNA *in vivo* or *in vitro* [49], it is possible that PTU is desulfurated to a uridine derivative which could then counteract the D-GNH_2-induced depletion of uridine nucleotides noted above. This, too, is unlikely since thiouracil conversion to uracil does not occur in rat liver [49]. Another possibility is that PTU acts either by serving as a source of necessary nonprotein sulfhydryl groups or by preserving intracellular GSH concentrations. Finally, since an increase in intracellular calcium has been observed in several models of experimental hepatic necrosis, including D-GNH_2 [50], it is also possible that PTU has effects on calcium transport into hepatocytes.

Structure–activity studies were undertaken in order to localize the site on the PTU molecule responsible for its protective effects. A free or unstable sulfhydryl group (as in PTU, *S*-methyl PTU, and 1-methyl-mercaptoimidazole) is necessary for activity, since propyluracil, which lacks an -SH group, was obviously ineffective at the dose that was utilized. However, a sulfhydryl is not sufficient, as 2-thiouracil and 2-thiobarbituric acid were also without effect at the dose that was employed, possibly due to the absence of the lipophilic propyl group. Surprisingly, *S*-methyl-6-PTU was quite effective in our system, even though this compound lacks a free thiol group. It is likely that *S*-methyl-6-PTU is demethylated *in vivo* to form PTU [51]. Recently, two other thiol-containing compounds, *S*-adenosylmethionine [52] and α,α-dithio-dicapronic acid [53] have also been reported to protect against D-GNH_2 liver injury.

In summary, PTU reduced the degree of liver injury associated with D-GNH_2 administration. This effect is probably mediated at the level of the liver, with the sulfhydryl group and the propyl group both being important for activity. Future studies will be necessary to elucidate the precise mechanism by which PTU exerts its protective effects.

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