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METABOLIC ACTIVATION OF THE TRICYCLIC ANTIDEPRESSANT AMINEPTINE—II

PROTECTIVE ROLE OF GLUTATHIONE AGAINST *IN VITRO* AND *IN VIVO* COVALENT BINDING

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Abstract—Incubation of [11-^{14}C]amineptine (1 mM) with an NADPH-generating system and hamster liver microsomes resulted in the *in vitro* covalent binding of an amineptine metabolite to microsomal proteins; this binding was decreased by 41–71% in the presence of cysteine, lysine, glycine or glutathione (0.5 mM). An inverse relationship was found between the concentration of glutathione in the incubation mixture (0.25–4 mM) and the extent of covalent binding *in vitro*, which became undetectable at concentrations of glutathione of 2 mM and higher. Administration of [11-^{14}C]amineptine (300 mg/kg^{-1} i.p.) to hamsters pretreated with phorone (500 mg/kg i.p.) resulted in the *in vivo* covalent binding of an amineptine metabolite to hepatic proteins. This binding was increased by phenobarbital-pretreatment and decreased by piperonyl butoxide-pretreatment. After various doses of phorone (150–500 mg/kg), an inverse relationship was found between hepatic glutathione content and *in vivo* covalent binding. Administration of amineptine alone (300 mg/kg i.p.) depleted hepatic glutathione by 16% only; in these animals, *in vivo* covalent binding was undetectable from background. Amineptine (300 mg/kg i.p.) did not produce hepatic necrosis, even in hamsters pretreated with phorone and/or phenobarbital. We conclude that physiologic concentrations of glutathione essentially prevent the *in vivo* covalent binding of an amineptine metabolite to hepatic proteins, and that this binding does not produce liver cell necrosis in hamsters.

Amineptine is a tricyclic anti-depressant widely used in several countries. Amineptine may produce hepatitis in some patients [1–3]. In the preceding communication, we report that amineptine is transformed by hamster liver and kidney microsomal cytochrome P-450 into a reactive metabolite which covalently binds to microsomal proteins *in vitro* [4]. In the present communication, we describe the protective role of glutathione against *in vitro* and *in vivo* covalent binding, and report histologic studies in hamsters.

MATERIALS AND METHODS

Animals and treatments. Male golden Syrian hamsters, weighing 90–110 g, were purchased from Fichot (Ormesson, France). Animals were fed *ad libitum* with a normal standard diet (M_{25} biscuits, Extralabo, Provins, France).

Amineptine hydrochloride and [11-^{14}C]amineptine hydrochloride were generously given by Servier Laboratories (Neuilly, France). The radiochemical, labelled on the tricyclic ring, was prepared by Commissariat à l'Energie Atomique, Gif-sur-Yvette, France. Its radiochemical purity was found to be >99% by HPLC.

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Amineptine hydrochloride (300 mg/kg) or [11-^{14}C]amineptine hydrochloride (0.1 mCi/kg, 300 mg/kg) were administered i.p. dissolved in 1 ml of 0.1 N NaOH. The final pH of the solution was 8.5. Some animals were pretreated with phenobarbital (100 mg/kg i.p. in 0.5 ml of water) daily for 3 days, and were used 24 hr after the last dose. Other animals received piperonyl butoxide (400 mg/kg i.p. given in 0.3 ml of corn oil) 1 hr before the administration of amineptine. Some animals were treated with buthionine sulphoximine (800 mg/kg i.p., given in 0.8 ml of 0.1 N NaOH) 4 hr before the administration of amineptine. Other hamsters received phorone (150–500 mg/kg i.p.) given in 0.5 ml of corn oil, 30 min before the administration of amineptine. Administration of phorone (isopropylideneacetone) has been shown to markedly decrease hepatic glutathione concentration [5].

In vitro *covalent binding*. Preparation of microsomes, measurement of proteins, and *in vitro* covalent binding studies were performed as reported in the preceding communication [4]. In some flasks, various concentrations of glutathione (0.25–4 mM) were added to the incubation mixture. In other flasks, various other nucleophiles (0.5 mM) were added.

In vivo *covalent binding*. Hamsters were killed at various times after administration of [11-^{14}C]amineptine (0.1 mCi/kg, 300 mg/kg i.p.). The liver, kid-

neys, lungs and a muscle fragment were removed and homogenized in 3 volumes of 0.154 M NaCl. The amount of ^{14}C irreversibly bound to proteins in the homogenates was then determined as previously described [4, 6–8]. Briefly, proteins were precipitated into filter paper disks (Durieux 111) that had been soaked in 5% trichloroacetic acid and had been dried at room temperature. After drying, the disks and their entrapped proteins were then washed three times with trichloroacetic acid, and repeatedly extracted with various solvents (methanol, twice; *n*-heptane, once; ether, once). We verified that radioactivity could not be removed further by introducing additional solvent extractions. The filter paper disks were then placed on the bottom of scintillation vials; 10 ml of scintillation fluid was added and ^{14}C was counted with background and quench correction. We verified [4] that [11-^{14}C]amineptine did not bind to the filter paper itself and that essentially similar values were obtained when proteins were counted while still entrapped in the filter paper disks, or after being first dissolved in 1 N NaOH (followed by neutralization with HCl). We also verified that this filter paper technique gave results essentially identical to those obtained when similar washings and extractions were performed on proteins remaining free in the test tube.

Other studies. Hepatic or renal glutathione concentration was determined as total non-protein sulphydryl groups by the method of Ellman [9]. Glutathione disulphide was determined as previously reported [10]. Benzo[a]pyrene hydroxylase and 7-ethoxycoumarin deethylase activities were measured as described by Kuntzman *et al.* [11], and Greenlee and Poland [12], respectively; the substrates (0.25 mM) were incubated for 10 min with hepatic microsomes and an NADPH-generating system.

Some liver fragments were placed in Bouin's fluid, embedded in paraffin 24 hr later, cut and stained with hematoxylin and eosin, and examined for the presence of liver cell necrosis.

Statistical analysis. Student's *t*-test for independent data was used to assess the significance of differences between means when only one comparison had been performed in the experimental protocol. Analysis of variance and Dunnett's test were used whenever a single control group had been compared to several other groups in the experimental protocol.

RESULTS

Protective effects of glutathione and other nucleophiles on in vitro *covalent binding*

Incubation of [11-^{14}C]amineptine with hamster liver microsomes and an NADPH-generating system, under air, for 5 min resulted in the *in vitro* covalent binding of a [^{14}C]amineptine metabolite to microsomal proteins (Fig. 1). This binding was markedly reduced when various nucleophiles (glutathione, cysteine, lysine and glycine) were added, at a low concentration (0.5 mM), to the incubation mixture (Fig. 1). Of these nucleophiles, glutathione was the most active (Fig. 1). Glutathione, cysteine, S-methylglutathione, lysine and glycine (0.5 mM) did not modify the 7-ethoxycoumarin deethylase and benzo[a]pyrene hydroxylase activities of hepatic microsomes from phenobarbital-pretreated hamsters (not shown).

Figure 2 shows the *in vitro* covalent binding of an amineptine metabolite to microsomal proteins in the presence of various concentrations of exogenous glutathione. As the concentration of glutathione was raised from 0 to 1 mM, there was a precipitous fall in the amount of covalently bound metabolite (Fig. 2). No significant covalent binding could be detected when the concentration of glutathione was 2 mM or higher (Fig. 2).

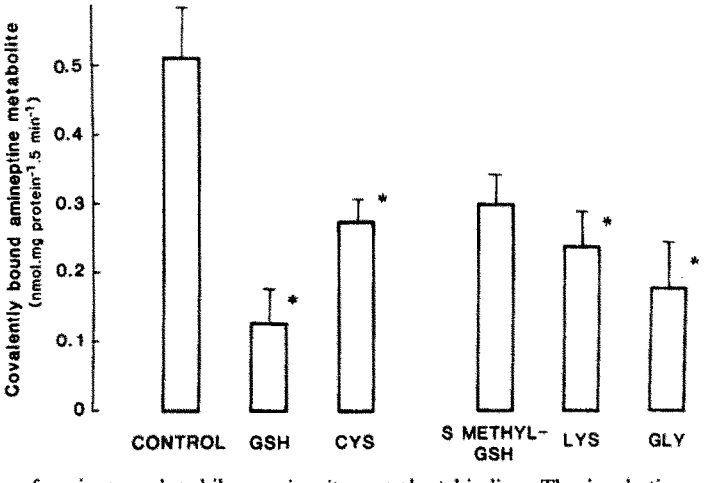

Fig. 1. Effects of various nucleophiles on *in vitro* covalent binding. The incubation mixture (1 ml) contained [11-^{14}C]amineptine (1 μCi/ml, 1 mM), an NADPH-generating system (1 mM NADP) and pooled hepatic microsomes from phenobarbital-pretreated hamsters (6 mg of microsomal proteins per ml). In some flasks, various nucleophiles (0.5 mM) were added. After 5 min of incubation at 37°, under air, the amount of ^{14}C irreversibly bound to microsomal proteins was determined. Results are means ± SEM for four determinations. The asterisks indicate a significant difference from the value in the absence of added nucleophile, $P < 0.05$.

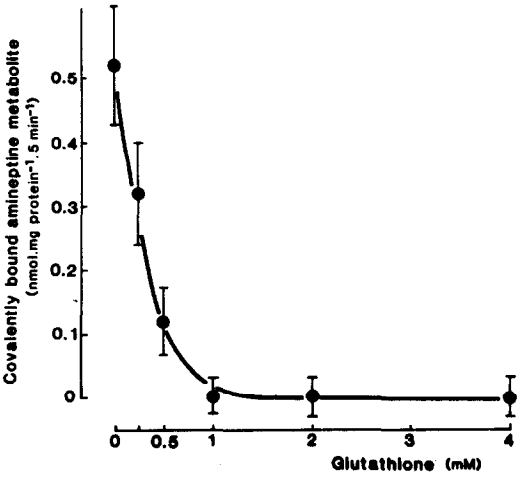

Fig. 2. Inverse relationship between the concentration of glutathione and *in vitro* covalent binding. The incubation mixture (1 ml) contained [11-^{14}C]amineptine (1 μCi/ml, 1 mM), an NADPH-generating system (1 mM NADP), and pooled hepatic microsomes from phenobarbital-pretreated hamsters (6 mg of microsomal proteins per ml). In some flasks, glutathione was added at various concentrations (0.25–4 mM). After 5 min of incubation under air at 37°, the amount of ^{14}C irreversibly bound to microsomal proteins was determined. Results are means ± SEM for six determinations.

Protective effects of glutathione on in vivo *covalent binding*

Figure 3 shows the amount of ^{14}C irreversibly bound to tissue proteins after administration of [11-^{14}C]amineptine (300 mg/kg i.p.). In animals not pretreated with phorone, no significant covalent binding of a metabolite could be detected. Indeed, only traces of radioactivity were found in tissue proteins (Fig. 3). This background labelling occurred almost instantaneously, was essentially similar in all organs tested, and was unaffected by pretreatment of the animals with piperonyl butoxide or phenobarbital (Fig. 3); it could not be decreased by administering [11-^{14}C]amineptine mixed with an equimolar dose of glutathione (to trap any reactive impurity). In contrast, in animals pretreated with phorone (500 mg/kg i.p. 30 min before the administration of amineptine), covalent binding was now clearly detectable (Fig. 3). The amount of covalent binding followed the order of cytochrome P-450 content in each organ, being maximum in the liver, intermediate in kidney and not clearly detectable in lung or muscle (Fig. 3). Covalent binding in liver and kidney increased progressively with time, reaching a maximum 2 hr after the administration of amineptine (Fig. 3). Pretreatment with piperonyl butoxide decreased covalent binding in liver and kidney (Fig. 3). Pretreatment with phenobarbital increased *in vivo* covalent binding in the liver but not in the kidney (Fig. 3). Covalent binding in hamsters pretreated with phorone affected similarly microsomal and cytosolic proteins of the liver: 2 hr after administration of [11-^{14}C]amineptine (0.1 mCi/kg; 300 mg/kg i.p.), 0.70 ± 0.06 nmol of amineptine metabolite was bound to microsomal proteins (mean ± SEM for six hamsters), and 0.95 ± 0.16 nmol was bound to cytosolic proteins.

In animals pretreated with various doses of phorone (150–500 mg/kg i.p. 30 min before the administration of amineptine), an inverse relationship was found between the concentration of glutathione in tissue and the extent of *in vivo* covalent binding in liver and kidney (Fig. 4).

Effect of amineptine administration on hepatic glutathione levels

Hepatic glutathione concentration was decreased slightly (by 16%), 2–6 hr after the administration of amineptine (Fig. 5). Glutathione disulphide concentration in the liver was not significantly modified (not shown). To decrease the synthesis of glutathione and sensitize the animals to the depleting effects of amineptine, we administered buthionine sulphoximine (800 mg/kg i.p.), 4 hr before the administration of amineptine (Fig. 6). As compared to that in animals receiving buthionine sulphoximine only, the per cent loss of hepatic glutathione in animals also treated with amineptine 2 hr earlier was now 40% (Fig. 6); glutathione disulphide content was not significantly modified, being 6.1 ± 0.5 nmol/g liver after buthionine sulphoximine alone and 5.7 ± 0.3 in animals receiving in addition amineptine 2 hr earlier (mean ± SEM for five hamsters). The loss of hepatic glutathione was smaller (20%) in animals pretreated with piperonyl butoxide (Fig. 6), and tended to be higher (51%) in animals pretreated with phenobarbital (Fig. 6).

Liver histology

Liver histology was normal in all five hamsters killed 24 hr after the administration of amineptine (300 mg/kg i.p.), as well as in five animals pretreated with phenobarbital and killed 24 hr after the administration of amineptine. Hamsters pretreated with phorone (500 mg/kg i.p.) before the administration of amineptine (300 mg/kg i.p.) died between 6 and 24 hr after the administration of amineptine, whereas hamsters receiving only phorone survived. No liver cell necrosis, however, could be detected in any of five phorone-pretreated hamsters sacrificed 6 hr after the administration of amineptine (including one that died spontaneously at that time), showing that death was not due to liver necrosis. Liver necrosis was also absent in five hamsters pretreated with both phenobarbital and phorone, and killed 6 hr after the administration of amineptine (300 mg/kg i.p.).

DISCUSSION

In the preceding communication [4], amineptine has been shown to be transformed by cytochrome P-450 into a chemically reactive metabolite that covalently binds to microsomal proteins *in vitro*. The present investigation shows the protective role of glutathione in this system, as demonstrated by the inverse relationship between the concentration of glutathione and the extent of covalent binding both *in vitro* (Fig. 2) and *in vivo* (Fig. 4). *In vitro*, the

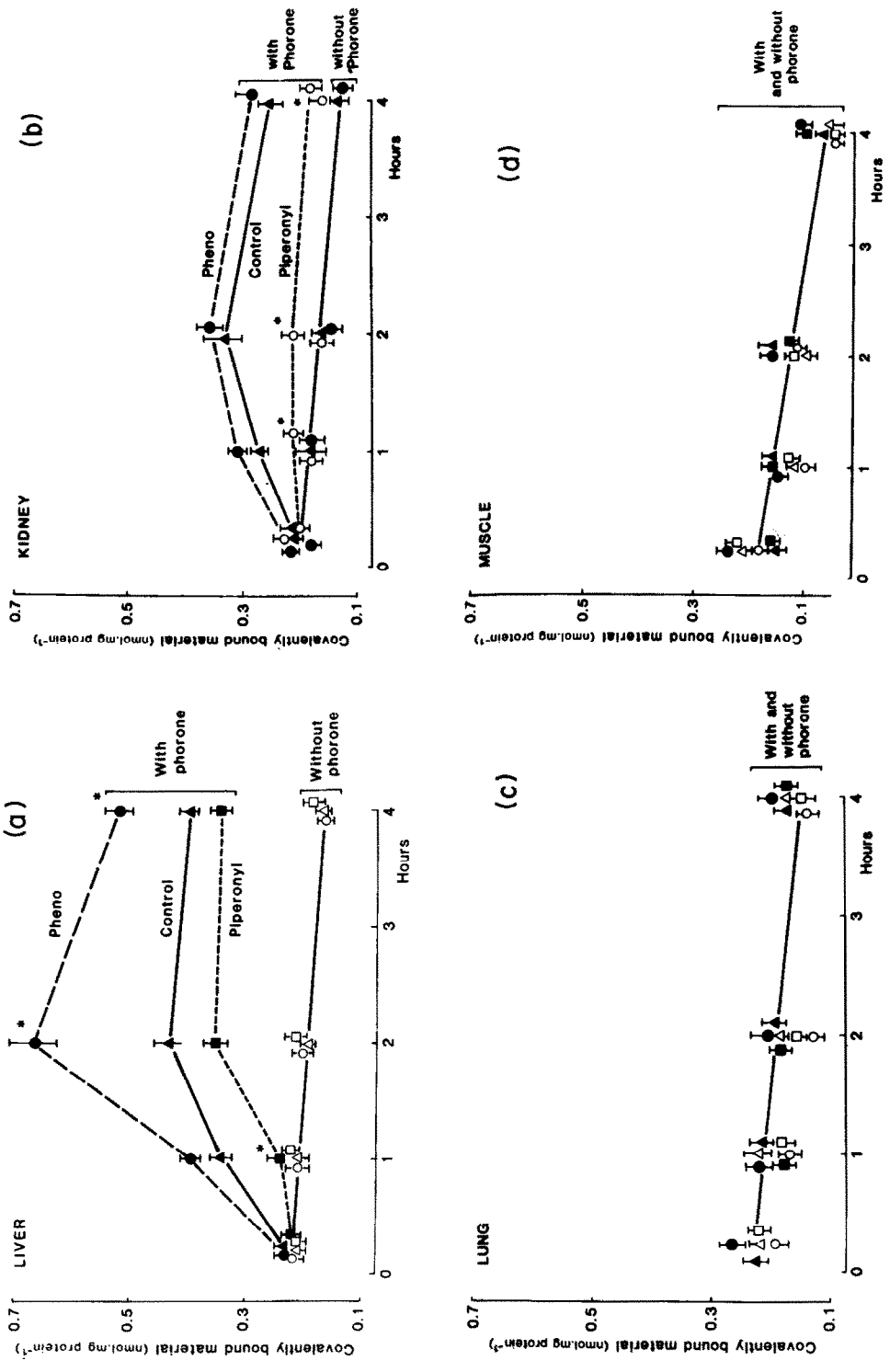

Fig. 3. Effects of various pretreatments on *in vivo* covalent binding. Hamsters received one of the following pretreatments: ●- -● phenobarbital (100 mg/kg i.p. daily for 3 days) + phorone (500 mg/kg i.p., 30 min before amineptine); ▲——▲ phorone only; ■- -■ piperonyl butoxide (400 mg/kg i.p. 60 min before amineptine) + phorone; ○——○ phenobarbital; △——△ no pretreatment; □——□ piperonyl butoxide. Animals were killed at various times after the administration of [11-^{14}C]amineptine (0.1 mCi/kg; 300 mg/kg i.p.) and the amount of ^{14}C irreversibly bound to proteins in various organs was determined. Results are means ± SEM for six hamsters. The asterisks indicate significant differences from values in hamsters pretreated with phorone only, $P < 0.05$.

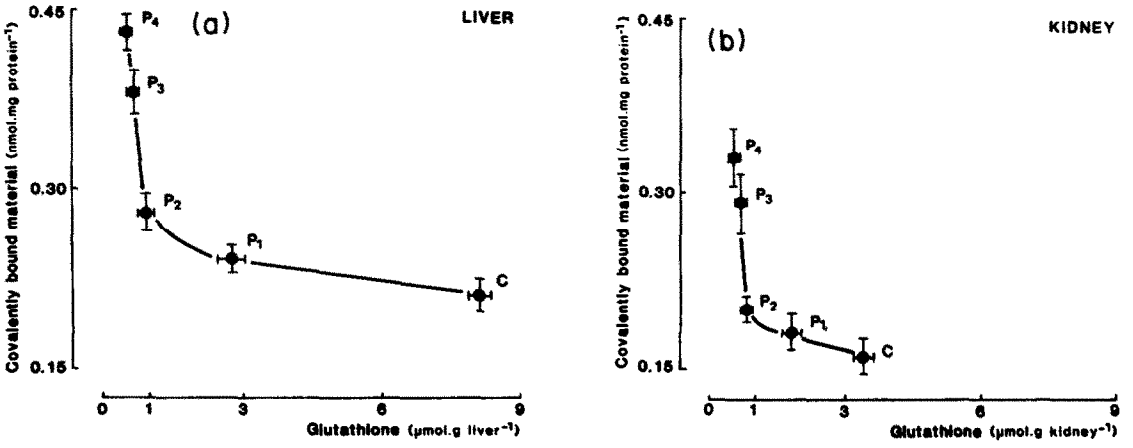

Fig. 4. Inverse relationship between the concentration of glutathione and *in vivo* covalent binding. Some hamsters were not pretreated (C), while others received various doese of phorone i.p. (P_1, 150 mg/kg; P_2, 350 mg/kg; P_3, 400 mg/kg; P_4, 500 mg/kg) 30 min before the administration of amineptine. Hepatic or renal glutathione was determined as total non-protein sulphydryls 1 hr after the administration of amineptine (300 mg/kg i.p.). Irreversible binding to liver or kidney proteins was determined 2 hr after the administration of [11-^{14}C]amineptine (0.1 mCi/kg; 300 mg/kg i.p.). Results are means ± SEM for four hamsters.

protective effect of glutathione was reproduced not only by cysteine but also by glycine and lysine (Fig. 1), suggesting that the reactive metabolite of amineptine may react not only with SH groups but also with slightly harder nucleophiles [13], such as the amine groups of proteins. A protective role of glutathione has been reported with many other chemically reactive metabolites [14, 15]. Usually, however, this protection is only partial. Detectable covalent binding still occurs *in vitro* and *in vivo* in the presence of physiologic concentrations of glutathione [14–21].

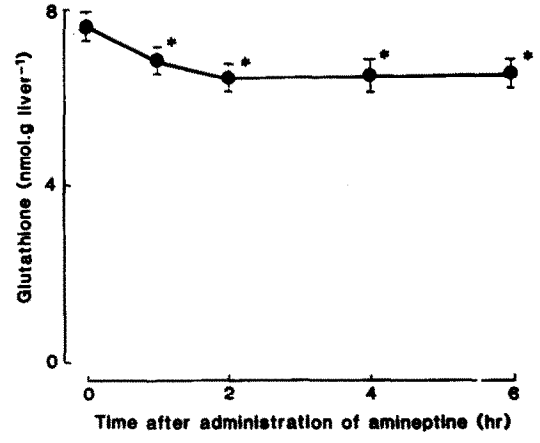

Fig. 5. Time course of hepatic glutathione concentration after administration of amineptine. Hamsters were killed at various times after the administration of amineptine (300 mg/kg i.p.) and the concentration of glutathione in the liver was determined as total non protein sulphydryls. Results are means ± SEM for eight hamsters. The asterisks indicate significant differences from values in concomitantly killed untreated hamsters, $P < 0.01$.

In the case of amineptine, however, the protection afforded by glutathione was almost complete. *In vitro*, concentrations of glutathione of 2 mM and higher (Fig. 2) decreased covalent binding down to levels undectable from background radioactivity in the zero-time sample. Similarly *in vivo*, covalent binding remained undetectable from background radioactivity, unless the concentration of glutathione had been depleted by previous administration of phorone (Figs. 3, 4).

In such phorone-pretreated animals, covalent binding was maximum in the liver, intermediate in kidney, and not detectable in lung or muscle. This distribution appears to follow the order of cytochrome P-450 contents in these organs [22] and is a first evidence for the *in situ* covalent binding of the reactive amineptine metabolite formed in each organ. Further evidence is provided by different effects of pretreatments in liver and kidney. Piperonyl butoxide, which prevented *in vitro* metabolic activation in both liver, and kidney microsomes [4], prevented *in vivo* covalent binding both in liver and in kidney (Fig. 3). In contrast, phenobarbital which was a poor inducer of kidney cytochrome P-450 and increased *in vitro* metabolic activation by liver microsomes but not by kidney microsomes [4], increased *in vivo* covalent binding in the liver but not the kidney (Fig. 3). These observations may indicate that the reactive metabolite that covalently binds to kidney proteins is *mainly* formed *in situ* in the kidney.

Conjugation and/or reduction of reactive metabolites by glutathione may consume and deplete hepatic glutathione [14, 15]. Indeed, administration of amineptine did decrease hepatic glutathione (Fig. 5). This depletion of glutathione was probably due to an amineptine metabolite, since it was partly prevented by pretreatment with piperonyl butoxide (Fig. 6). Metabolic activation, however, is probably a

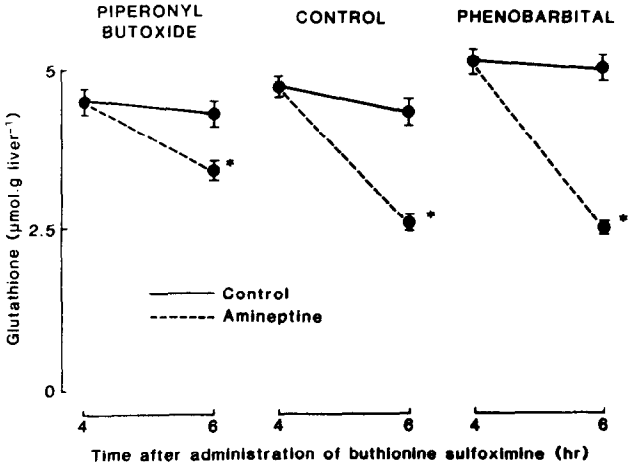

Fig. 6. Effects of pretreatments on the depletion of glutathione 2 hr after the administration of amineptine. All hamsters received buthionine sulphoximine (800 mg/kg i.p.) 4 or 6 hr before being killed. Some hamsters ("amineptine") received amineptine (300 mg/kg i.p.) 4 hr after the administration of buthionine sulphoximine, while others ("control") did not. Some hamsters ("CONTROL") received no other pretreatment, while others ("PHENOBARBITAL") had been pretreated for 3 days with phenobarbital (100 mg/kg i.p. daily); still other hamsters ("PIPERONYL BUTOXIDE") received piperonyl butoxide (400 mg/kg i.p.) 3 hr after the administration of buthioxine sulphoximine. Hepatic glutathione concentration was measured as total non-protein sulphydryls. Results are means ± SEM for six hamsters. Asterisks indicate significant differences from values in hamsters not treated with amineptine, $P < 0.05$.

minor pathway only in the metabolism of amineptine [4]. The depletion of glutathione after administration of amineptine remains small (Fig. 5), unable to affect significantly *in vivo* covalent binding (Figs. 2–4).

From our observations in hamsters, it is tempting to speculate on a possible mechanism for amineptine hepatitis in humans. In hamsters, administration of amineptine, at the dose of 300 mg/kg, failed to produce hepatic necrosis, even in animals pretreated with both phenobarbital and phorone. In humans, in whom the doses of amineptine are 100-times smaller, hepatitis occurring after therapeutic doses of amineptine may not be toxic in type [1–3]. Instead, it has several features suggesting an allergic mechanism [1–3]. Conceivably, low levels of covalent binding may trigger, in a few subjects, an immunisation against plasma membrane macromolecules altered by the covalent binding of the metabolite. Such a metabolite-triggered allergic mechanism is currently proposed for hepatitis produced by halothane [23], isaxonine [16, 17], α-methyl dopa [24], and imipramine [25], another tricyclic anti-depressant drug. Many other drugs are thought to be hepatotoxic through allergic mechanisms [26], but we still do not know whether they are transformed or not into reactive metabolites. By testing at least a few of these drugs, we should be able to tell whether the formation of a reactive metabolite is a prerequisite (or a favouring factor) for drug-induced allergic hepatitis to occur.

We conclude that physiologic concentrations of glutathione essentially prevent the *in vivo* covalent binding of an amineptine metabolite to hepatic proteins and that this binding does not produce liver cell necrosis in hamsters.

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