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# MORPHINE METABOLISM IN ISOLATED RAT HEPATOCYTES AND ITS IMPLICATIONS FOR HEPATOTOXICITY

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**Abstract**—Isolated rat hepatocytes metabolized morphine to its glucuronide conjugate, morphinone–glutathione conjugate, normorphine and morphinone. Addition of morphine to the isolated hepatocytes induced a marked decrease in the level of glutathione in the cells and resulted in cell death. The formation of glutathione conjugate was correlated well with the loss of intracellular glutathione. The cytotoxicity of morphinone was higher than that of morphine. Naloxone and normorphine showed no cytotoxic effect on the cells. Naloxone inhibited the formation of morphinone–glutathione conjugate and prevented the morphine-induced cytotoxicity. Naloxone also blocked morphine-induced liver damage *in vivo*. In contrast, the morphinone-induced hepatotoxicity was not prevented by naloxone. It is concluded that morphine has a hepatotoxic effect, that the morphine-induced hepatotoxicity is due to its metabolic activation, and that naloxone acts as an inhibitor of an enzyme converting morphine to morphinone.

The pathogenesis of liver dysfunction among narcotic addicts has been debated for many years. Previous studies have established that a large dose of administered morphine results in an acute depletion of hepatic glutathione (GSH) [1–3] as well as elevations of the serum glutamic oxaloacetic transaminase (GOT) and serum glutamic pyruvic transaminase (GPT) activities [2–5]. Morphological findings have also demonstrated that the liver is filled with round lipid droplets after morphine administration [6, 7]. Chang and Ho [4] and Needham *et al.* [7] indicated that a central nervous system mediated action of morphine is involved in the hepatotoxicity. Recently, Correia *et al.* [3] proposed that hepatic microsomal cytochrome P-450 is linked to the toxicity. We have also suggested that the formation of morphinone by cytoplasmic morphine 6-dehydrogenase might be involved in morphine-induced hepatotoxicity [1].

Suspensions of freshly isolated hepatocytes have become increasingly used as an experimental model for studies on the mechanisms of various drug- and xenobiotic-induced toxicities [8–10]. In this paper, we examine the metabolism of morphine in freshly isolated rat hepatocytes as well as some results related to *in vitro* and *in vivo* mechanisms of morphine-induced hepatotoxicity.

## MATERIALS AND METHODS

*Chemicals.* Naloxone was a gift from the Sankyo Co. Ltd., Tokyo, Japan. Morphinone and dihydromorphinone were prepared by the method of Rapoport *et al.* [11, 12]. Normorphine was synthesized by the method of Braun [13]. Collagenase was obtained from Boehringer/Mannheim GmbH, West Germany. All other reagents were of the analytical grade commercially available.

*Hepatocyte preparation and metabolism of morphine.* Male Wistar rats, weighing 200–250 g, were used. Isolation of hepatocytes was performed by collagenase perfusion as described previously [9]. The yield of each preparation was 2–4 × $10^8$ cells/rat. The cell viability immediately after the isolation was 90–95% by the trypan blue exclusion method.

Incubations of hepatocytes were performed at 37° in rotating round-bottom flasks [14] under a 95% $O_2$–5% $CO_2$ atmosphere, at a cell concentration of $10^6$ cells/ml, in Krebs–Henseleit buffer, pH 7.4. Morphine hydrochloride was dissolved in Krebs–Henseleit buffer and added to the incubation mixture.

Metabolites of morphine were analyzed by high performance liquid chromatography (HPLC) (Toki *et al.*, personal communication). The reaction was terminated by removing the hepatocytes from the incubation mixture by centrifugation at 600 *g* for 3 min, and the supernatant fraction was used for metabolite analysis immediately or after storage at −20°. An aliquot (50 μl) of the supernatant fraction was injected onto the HPLC column. The HPLC system was equipped with a Shimadzu LC-3A pump, a Rheodyne sample injector, a Waters Radial-pak C-8 column (8 mm × 10 cm) and a Shimadzu SPD-2A variable wavelength u.v. detector. The column was eluted with water/acetonitrile/triethylamine/acetic acid (190:10:1:1, by vol.) at a flow rate of 2 ml/min. The eluate was monitored at 280 nm.

*Cell viability.* The cell viability was calculated from the activity of lactate dehydrogenase leaked from damaged cells [14]. The viability measured by this

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method is known to be in good agreement with that obtained by the trypan blue exclusion method [14]. An aliquot of a well-mixed hepatocyte suspension was diluted 60-fold in Krebs–Henseleit buffer, and NADH and pyruvate (0.2 and 1.5 mM as final concentrations respectively) were added. The rate of NADH oxidation by the enzyme was measured from the decrease in absorbance at 340 nm. The enzymatic activity after lysis of the cells by the addition of Triton X-100 (1.0% as final concentration) was taken as the total activity.

*Measurement of GSH.* The intracellular level of GSH was estimated routinely by the method of Hissin and Hilf [15]. An aliquot of the deproteinized cell suspension (50 μl) was mixed with 2.1 ml of 200 mM sodium phosphate buffer, pH 8.0, containing 5 mM sodium ethylenediamine tetraacetic acid. Then, 100 μl of a solution of *o*-phthalaldehyde (1 mg/ml in methanol) was added, and 15 min later the intensity of fluorescence was determined (excitation 350 nm, emission 420 nm). The intracellular GSH level was also confirmed by the HPLC method [16]. The values obtained by both methods coincided well with each other.

Hepatic GSH (non-protein sulfhydryl compounds) was measured according to the method described by Ellman [17] with slight modifications. Liver homogenates (20%) in 5% (final) trichloroacetic acid containing 1 mM sodium ethylenediamine tetraacetic acid were centrifuged at 2000 *g* for 5 min. An aliquot of the supernatant fraction (400 μl) was transferred to a tube containing 4.55 ml of 0.1 M sodium phosphate buffer, pH 8.0, and 50 μl of 0.1 M 5,5-dithiobis(2-nitrobenzoic acid) was added to the mixture. After mixing, the absorbance at 410 nm was measured against a reagent blank to determine the GSH concentration. Reduced GSH was used to establish a standard curve.

*Effects of morphine, morphinone and naloxone on serum GOT and GPT activities in rats.* Five male rats in each group were given a single s.c. dose of morphine (100 mg/kg), morphinone (30 mg/kg) and/or naloxone (20 mg/kg). In the case of combinations with naloxone, naloxone was injected i.p. 10–15 min before the treatment with morphine or morphinone. All animals were killed at 3 hr after the administration. Blood was collected from the animals after decapitation. Serum was collected after clotting the blood by centrifugation at 2000 *g* for 15 min. The serum GOT and GPT activities were determined using reagent kits purchased from the Wako Chemical Co., Tokyo, Japan. To 500 μl of substrate solution (2 mM α-ketoglutarate and 200 mM alanine or 200 mM asparaginic acid in 0.1 M phosphate buffer, pH 7.4), a 100-μl aliquot of serum or saline was added and the reaction mixture was incubated at 37°. After 30 min, the reaction was terminated by the addition of 500 μl of color reagent (20%, 2,4-dinitrophenylhydrazine in 1 N HCl). The color was allowed to develop at room temperature for 20 min before the addition of 5 ml of 0.4 N NaOH. The intensity of the resultant color was measured with a spectrophotometer at 505 nm.

## RESULTS

*Identification of morphine metabolites.* Morphine was incubated with hepatocytes, and its metabolites in the incubation medium were analyzed by HPLC under the conditions described in 'Materials and Methods'. A typical HPLC profile of the incubation medium at 60 min (Fig. 1B) indicated the appearance of new peaks of metabolite (a, b, c and e) when compared with that of the medium obtained at 0 min (Fig. 1A). Under the conditions used, authentic morphinone, morphine, normorphine, morphinone–glu-

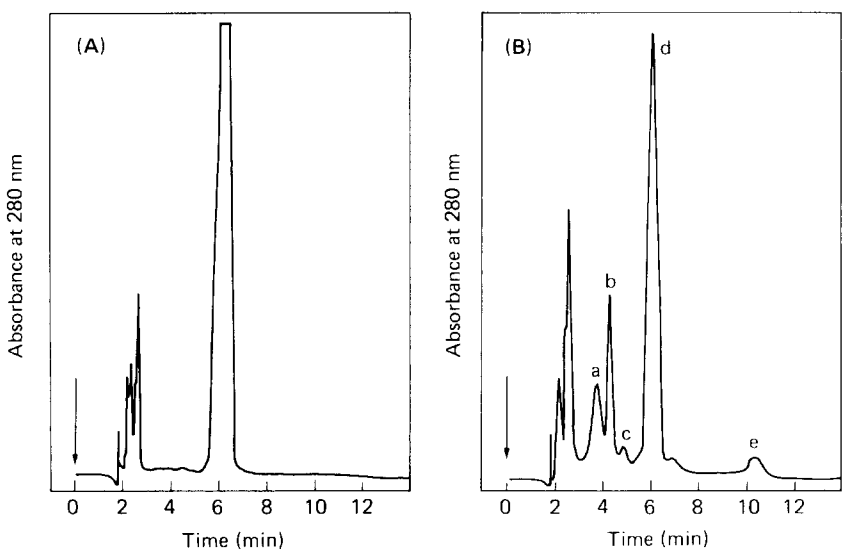


Fig. 1. HPLC profiles of morphine metabolites produced by isolated rat hepatocytes. The incubation mixtures at 0 (A) and 60 (B) min were analyzed by HPLC as described in Materials and Methods. The morphine concentration was 250 μM. Key: (a) morphine glucuronide; (b) morphinone–glutathione conjugate; (c) normorphine; (d) morphine; and (e) morphinone.

tathione conjugate and morphine–glucuronide conjugate were eluted at 10.5, 6.2, 4.9, 4.3 and 3.8 min respectively. It was concluded, therefore, that the new peaks represented morphine glucuronide (peak a), morphinone–glutathione conjugate (peak b), normorphine (peak c), morphine (peak d) and morphinone (peak e) respectively. Furthermore, peak b, eluting at 4.3 min, was confirmed to be morphinone–glutathione conjugate by NMR analysis (Toki *et al.*, personal communication). In these experiments, we employed the supernatant fraction of the incubation medium obtained by removal of the hepatocytes by centrifugation (600 *g*, 3 min). For comparison, the cells were first lysed in the incubated medium by freeze-thawing, and the supernatant fraction was then collected by centrifugation (2000 *g*, 10 min). The results obtained with both samples were essentially the same (data not shown).

*Cell damage associated with morphine metabolism.* Isolation of rat hepatocytes by liver perfusion with collagenase yielded viable cells in high quantity. Close to 100% of the freshly isolated cells excluded trypan blue. The cell damage associated with morphine metabolism was monitored by the lactate dehydrogenase (LDH) test. On incubation of the hepatocytes with morphine (125–500 μM) for up to 2 hr, there were marked decreases in the cellular GSH level. The viability of the isolated hepatocytes and the cellular GSH level were decreased with an increase in morphine concentration (Fig. 2). At a high concentration (500 μM), the GSH level dropped to less than 10% of the control, and almost 100% of the hepatocytes were damaged after 2 hr of incubation. Figure 3 shows the correlation between morphinone–glutathione formation and GSH concentration in isolated rat hepatocytes after incubation with morphine (500 μM). These findings indicate a correlation between the decrease in intracellular GSH and the formation of morphinone–glutathione conjugate.

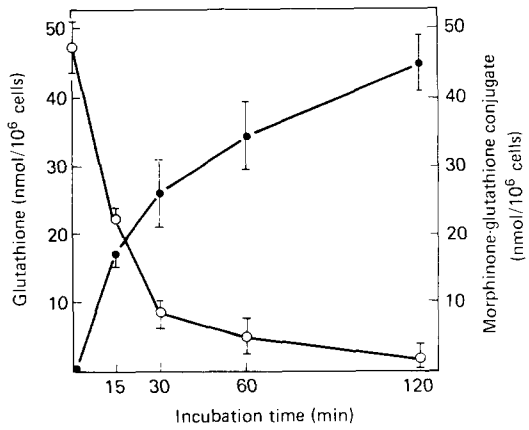


Fig. 3. Formation of morphinone–glutathione conjugate and decrease of GSH level in isolated rat hepatocytes. Isolated rat hepatocytes were incubated with 500 μM morphine as described in Materials and Methods. The data represent the mean ± S.D. of four experiments. Key: (●) morphinone–glutathione; and (○) glutathione.

*Effects of morphine, morphinone, dihydromorphinone and normorphine on cellular GSH levels and hepatocyte viability.* To clarify whether the morphine-induced decrease of cellular GSH level and the loss of membrane integrity were due to morphine or its metabolites, hepatocytes were incubated for 2 hr in the presence of morphine, morphinone, dihydromorphinone or normorphine, and cellular GSH levels and cell membrane integrity were monitored (Fig. 4). The control hepatocytes maintained a constant high level of GSH, and the cells showed no signs of loss of membrane integrity. In contrast, in the presence of morphinone (250 μM), the GSH

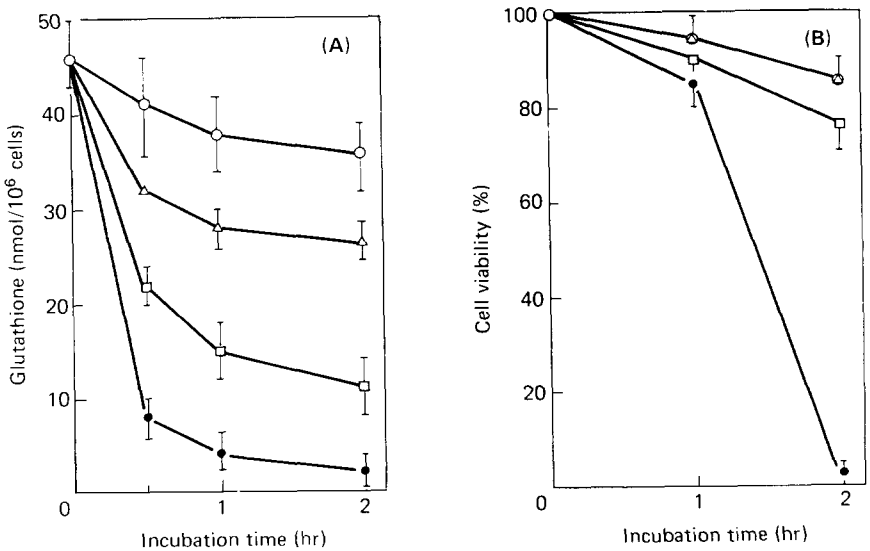


Fig. 2. Effects of morphine on cellular GSH (A) and viability (B). Isolated rat hepatocytes were incubated with various concentrations of morphine as described in Materials and Methods. The data represent mean ± S.D. of four experiments. Morphine: 0 μM (○), 125 μM (△), 250 μM (□), and 500 μM (●).

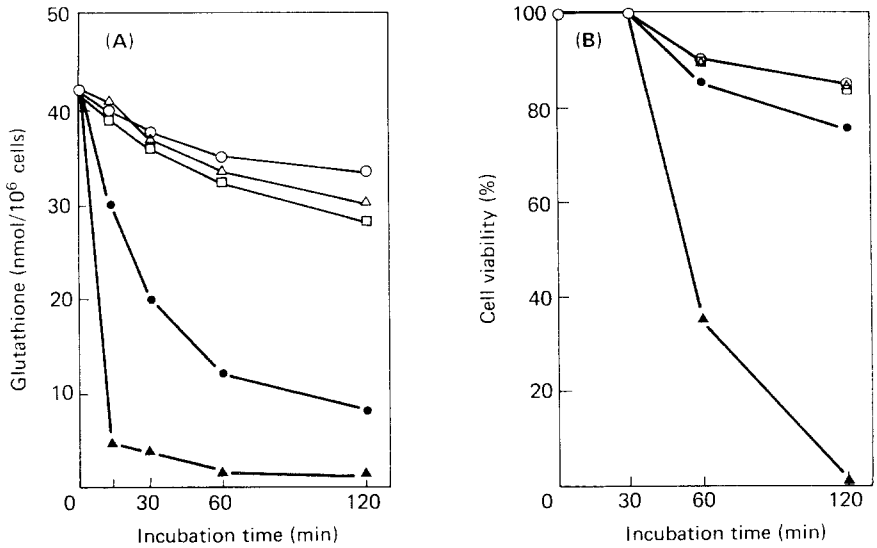


Fig. 4. Effects of morphine, morphinone, dihydromorphinone and normorphine on cellular GSH level (A) and viability (B). Isolated rat hepatocytes were incubated with each compound at a concentration of 250 μM. The data represent the mean of two experiments. Key: (○) control; (●) morphine; (▲) morphinone; (□) dihydromorphinone; and (△) normorphine.

level decreased immediately to about 5% of the control, and essentially no cells remained viable. Similarly, in the presence of morphine (250 μM), the cellular GSH level decreased with time and after 2 hr the hepatocytes exhibited an increased membrane permeability. Also, at this stage, more than 20% of the hepatocytes were damaged. Dihydromorphinone (250 μM), which cannot react non-enzymatically with GSH [1], did not deplete GSH in the isolated hepatocytes. Normorphine (250 μM) revealed no apparent effect on cellular GSH levels and cell viability. When a high concentration of morphine (500 μM) was used, more than 90% of the hepatocytes died within 2 hr. Dihydromorphinone (500 μM) and normorphine (500 μM) slightly lowered the cellular GSH level, but even after 3 hr there was no sign of any loss in cell viability (data not shown).

*Effects of naloxone on the formation of morphinone–glutathione conjugate in hepatocytes and morphine-induced cytotoxicity.* As shown in Figs. 1 and 3, morphine was metabolized to morphinone–glutathione conjugate in the isolated hepatocytes and, concomitantly, the intracellular GSH level was decreased with time. We therefore investigated the effects of naloxone, a potent antagonist of morphine, on the formation of morphinone–glutathione conjugate and morphine-elicited cytotoxicity. As shown in Fig. 5, naloxone markedly inhibited the formation of morphinone–glutathione conjugate. Consistent with this, naloxone almost completely abolished the cytotoxicity of morphine to the isolated hepatocytes (Fig. 6).

*Hepatotoxic effect after injection of morphine, morphinone, and/or naloxone.* To examine the implications of the results obtained in the *in vitro* experiments in relation to the *in vivo* hepatotoxicity of morphine, the effects of morphine and morphinone on the serum GOT and GTP and hepatic GSH levels were investigated after s.c. administration of these drugs to rats (Table 1). Morphine and morphinone significantly depleted the liver GSH and elevated the serum GOT and GPT activities in the rat. Contrary to this, naloxone alone exerted no effect on these variables. However, naloxone when administered in combination with morphine significantly attenuated the morphine-induced serum GOT and GPT activities and depletion of hepatic GSH. These results are broadly consistent with those reported previously [4, 5]. In contrast, the morphinone-induced elevation of serum GOT and GPT activities and depletion of hepatic GSH were not prevented by naloxone (Table 1).

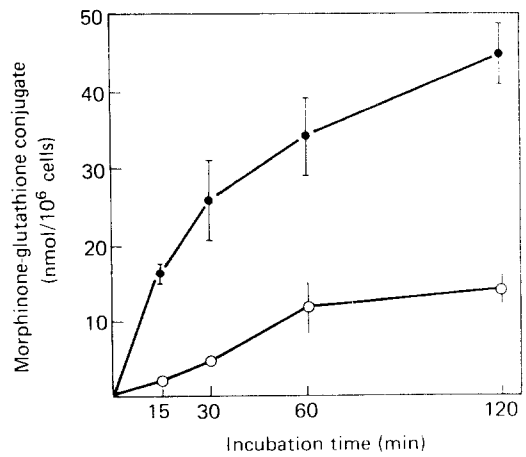


Fig. 5. Effect of naloxone on the formation of morphinone–glutathione conjugate from morphine in isolated hepatocytes. The data represent the mean ± S.D. of three experiments. Key: (●) morphine (500 μM); and (○) morphine (500 μM) + naloxone (500 μM).

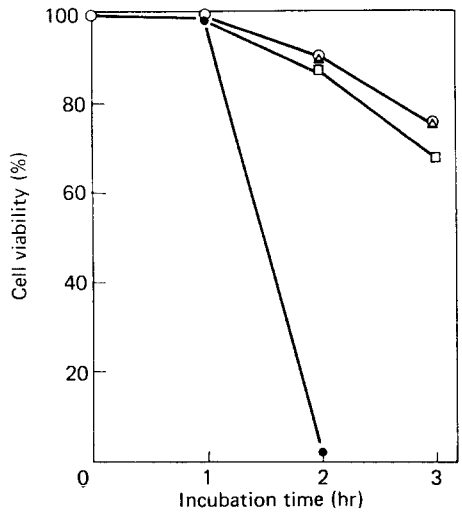


Fig. 6. Effect of naloxone on morphine-induced cytotoxicity. The data represent the mean of three experiments. Key: (○) control; (△) naloxone (500 μM); (□) morphine (500 μM) + naloxone (500 μM); and (●) morphine (500 μM).

## DISCUSSION

Freshly isolated hepatocytes represent an experimental model applicable to the investigation of drug metabolism linked cytotoxicity since the cells are capable of metabolic activation and/or inactivation of a variety of toxic agents and serve as a sensitive indicator system for various toxic effects.

In this study, we demonstrated the formation of morphine glucuronide, morphinone–glutathione conjugate, normorphine and morphinone when isolated rat hepatocytes were incubated with morphine. These metabolites were excreted into the incubation medium, and direct application of the medium to HPLC was able to separate and detect nmole quantities of the metabolites (Fig. 1).

GSH provides protection against reactive metabolites generated in the liver by acting as a scavenger of electrophilic intermediates which would otherwise react and inactivate tissue macromolecules essential to membrane integrity and cell viability [18, 19]. It has been shown that administration of a large dose of morphine causes depletion of hepatic GSH [1–3]. In this study, we also demonstrated that incubation of hepatocytes with morphine caused a rapid decrease in the intracellular GSH concentration and a marked loss of cell viability (Fig. 2). The rapid decrease in intracellular GSH was paralleled by the formation of morphinone–glutathione conjugate (Fig. 3). Morphine (500 μM) depleted about 40 nmoles/$10^6$ cells of GSH in hepatocytes after 30 min, whereas at this time, the amount of GSH conjugate formed by hepatocytes was only 25 nmoles/$10^6$ cells. Such a temporal discrepancy is due to the morphine-induced oxidative stress. This assumption is supported by the data that ethylmorphine depletes GSH in isolated hepatocytes and oxidizes GSH to glutathione disulfide (GSSG) [20]. Indeed, the amount of GSSG in morphine-treated hepatocytes was about 25 nmoles/$10^6$ cells higher than in control hepatocyte suspensions (data not shown). The GSSG is rapidly reduced back to GSH with a functioning glutathione reductase, since the amount of conjugate formed after 120 min (45 nmoles/$10^6$ cells) was comparable to that of the GSH present in the original cells (48 nmoles/$10^6$ cells). We have already shown that morphinone reacts non-enzymatically with glutathione to form morphinone–glutathione conjugate [1], and morphinone–glutathione conjugate has been recovered from the incubation mixture from mouse cytosol [21]. Moveover, morphinone-protein adduct has been detected in morphine-dosed mouse liver [21]. Morphinone was found to be more toxic to the hepatocytes and lowered the cellular GSH level more rapidly than morphine (Fig. 4). Morphinone also caused an accumulation of lipid in hepatocytes after s.c. treatment in the rat (Nagamatsu *et al.*, unpublished data). On the basis of these observations, we assumed that the morphine-induced hepatotoxicity might be due to the formation of morphinone, a reactive electrophilic intermediate, which attacks hepatocellular nucleophiles such as glutathione and proteins. Morphinone–glutathione conjugate was one of the major metabolites of morphine in the hepatocytes and was rapidly excreted into the extracellular space. Therefore, the formation of morphinone–glutathione conjugation via morphinone may represent one of the main detoxication pathways of morphine in the liver.

Table 1. Effects of morphine, morphinone and naloxone on serum GOT and GPT activities and hepatic glutathione content in rats

| Drug | Dose (mg/kg) | GOT (Karmen units/ml) | GPT (Karmen units/ml) | GSH (μmoles/g liver) |
|---|---|---|---|---|
| None (control) | | 115 ± 6 | 23 ± 0.7 | 4.13 ± 0.35 |
| Morphine | 100 | 136 ± 6* | 38 ± 4.9* | 3.09 ± 0.12* |
| Morphinone | 30 | 151 ± 8* | 36 ± 2.8* | 2.75 ± 0.20* |
| Naloxone | 20 | 119 ± 6 | 23 ± 1.8 | 3.83 ± 0.24 |
| Morphine + naloxone | 100<br>20 | 122 ± 4 | 20 ± 0.9 | 4.06 ± 0.16 |
| Morphinone + naloxone | 30<br>20 | 146 ± 6* | 33 ± 1.7* | 2.99 ± 0.07* |

Rats were treated with morphine, morphinone and/or naloxone, and killed at 3 hr following treatment. Serum GOT and GPT activities and hepatic glutathione content were determined as described in Materials and Methods. Each value represents the mean ± S.E. of five animals.

* Significance of the difference was calculated using Dunnett's test ($P < 0.05$).

The presence of naloxone in the incubation medium served to protect the cells from the morphine-induced cytotoxicity and decreased the formation of morphinone–glutathione conjugate in the isolated hepatocytes (Fig. 6). Since morphinone, once formed, reacts non-enzymatically with sulfhydryl compounds, these effects of naloxone were probably due to inhibition of the formation of morphinone. This assumption was supported by the fact that naloxone is an inhibitor of morphine 6-dehydrogenase, which catalyzes the dehydrogenation of morphine to morphinone [22].

Naloxone also prevented the morphine-induced glutathione depletion and elevation of serum GOT and GTP activities *in vivo* (Table 1). These results are consistent with those of previous *in vivo* studies which showed that naloxone prevents the morphine-induced increase of serum GOT and GPT levels [4, 5, 7]. In contrast, neither the depletion of GSH nor the elevation of serum GOT and GPT activities caused by morphinone was blocked by naloxone. Even though on the basis of these results we cannot comment about the central nervous system mediated hepatotoxicity of morphine, which was suggested by Chang and Ho [4] and Needham *et al.* [7], we clearly demonstrated that morphine can directly cause severe hepatocellular damage, and the present results support our previous conclusion that metabolic activation of morphine to morphinone in the liver represents one of the main causes of morphine-induced hepatotoxicity.

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