SYSTEMIC COADMINISTRATION OF CHLORAMPHENICOL WITH INTRAVENOUS BUT NOT INTRACEREBROVENTRICULAR MORPHINE MARKEDLY INCREASES MORPHINE ANTINOCICEPTION AND DELAYS DEVELOPMENT OF ANTINOCICEPTIVE TOLERANCE IN RATS

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ABSTRACT:
Chloramphenicol, an in vitro inhibitor of the glucuronidation of morphine to its putative antianalgesic metabolite, morphine-3-glucuronide (M3G), was coadministered with morphine in adult male Sprague-Dawley rats to determine whether it inhibited the in vivo metabolism of morphine to M3G, thereby enhancing morphine antinociception and/or delaying the development of antinociceptive tolerance. Parenteral chloramphenicol was given acutely (3-h studies) or chronically (48-h studies). Morphine was administered by the i.v. or i.c.v. route. Control rats received chloramphenicol and/or vehicle. Antinociception was quantified using the hotplate latency test. Coadministration of chloramphenicol with i.v. but not i.c.v. morphine increased the extent and duration of morphine antinociception by ~5.5-fold relative to rats that received i.v. morphine alone. Thus, the mechanism through which chloramphenicol enhances i.v. morphine antinociception in the rat does not directly involve supraspinal opioid receptors. Acutely, parenteral coadministration of chloramphenicol and morphine resulted in an ~75% increase in the mean area under the serum morphine concentration-time curve but for chronic dosing there was no significant change in this curve, indicating that factors other than morphine concentrations contribute significantly to antinociception. Antinociceptive tolerance to morphine developed more slowly in rats coadministered chloramphenicol, consistent with our proposal that in vivo inhibition of M3G formation would result in increased antinociception and delayed development of tolerance. However, our data also indicate that chloramphenicol inhibited the biliary secretion of M3G. Whether chloramphenicol altered the passage of M3G and morphine across the blood-brain barrier remains to be investigated.

Morphine is recommended by the World Health Organization as the drug of choice for the management of moderate to severe cancer pain (World Health Organization, 1986). However, chronic administration of morphine by systemic routes may result in the development of analgesic/antinociceptive tolerance, manifested as a diminution of the pain-relieving effect or the requirement for an increase in morphine dose to maintain satisfactory pain relief, without an underlying progression in the disease state.

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1 Parts of this research were presented in Abstract form at the National Scientific Conferences of ASCEPT (Australasian Society for Clinical and Experimental Pharmacologists and Toxicologists) and APSA (Australasian Pharmaceutical Sciences Association) in 1994 and 1996, respectively.

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Sprague-Dawley (SD)2 rats are commonly used for studies of morphine tolerance. In both rats and humans, more than half of every morphine dose is metabolized to morphine-3-glucuronide (M3G). However, in both SD and Wistar strains of rat (unlike humans), glucuronidation of morphine at the 6-position to form morphine-6-glucuronide (M6G), the analgesically active metabolite of morphine, does not occur in detectable quantities (Milne et al., 1996). Unlike M6G, M3G has no intrinsic analgesic/antinociceptive effects (Milne et al., 1996). Rather, after i.c.v. or intrathecal administration to rats, M3G has been shown to evoke a range of neuroexcitatory behavioral effects in a dose-dependent manner (Labella et al., 1979; Yaksh et al., 1986; Bartlett et al., 1994). Additionally, M3G administered supraspinally (but not spinally, Hewett et al., 1993; Suzuki et al., 1993) has been shown to markedly attenuate the antinociceptive effects of i.c.v. morphine (Smith et al., 1990) and i.c.v. M6G (Smith et al., 1990;
Gong et al., 1992; Faura et al., 1996), thereby suggesting that M3G is a putative antianalgesic metabolite of morphine.

Additionally, our studies (Smith and Smith, 1995) and those of Barjavel et al. (1995) have shown a highly significant inverse correlation ($r > 0.9$) between mean levels of antinociception (expressed as the percentage maximum possible effect, %MPE) achieved and the mean values of the molar concentration ratio, M3G/morphine, in the plasma and cortical extracellular fluid, respectively, for rats dosed with parenteral morphine. If this is the case, then in vivo inhibition of the metabolism of morphine to its putative antianalgesic metabolite, M3G, in the rat, should increase levels of antinociception and/or attenuate the development of antinociceptive tolerance to morphine.

A number of inhibitors of the glucuronidation of morphine to M3G have been identified in vitro and in vivo microsomal studies, including the clinically used drugs oxazepam, diazepam, chloramphenicol, several tricyclic antidepressants, fluoxetine, and the potentially toxic metal clinically used drugs oxazepam, diazepam, chloramphenicol, several tricyclic antidepressants, fluoxetine, and the potentially toxic metal ions, CD$^{2+}$, Cu$^{2+}$, and Cu$^{+}$ (Miners et al., 1988; Yue et al., 1990; Wahlstrom et al., 1994). Preliminary studies (data not shown) showed that oxazepam was unsuitable as an in vivo inhibitor of the metabolism of morphine to M3G because of its sedative effects, which would have confounded the interpretation of experiments involving its use. However, additional preliminary studies established that systemic administration of the antibiotic, chloramphenicol, also a competitive inhibitor of the in vitro glucuronidation of morphine to M3G (Miners et al., 1988), did not produce significant antinociception when administered alone to adult male SD rats in doses that significantly increased the antinociceptive effects of i.v. morphine. Therefore, this study was designed to investigate whether parenteral administration of chloramphenicol (inhibits glucuronidation of morphine to its putative antianalgesic metabolite, M3G) with: 1) i.v. morphine, would result in: a) increased levels of antinociception in adult male SD rats after both acute and chronic dosing, and/or b) attenuation of the development of antinociceptive tolerance in rats administered chronic i.v. morphine, relative to rats given chronic i.v. morphine alone; and 2) i.c.v. morphine, would alter the intrinsic antinociceptive effects of morphine.

### Experimental Procedures

**Animals.** Ethical approval was obtained from the Animal Experimentation Ethics Committee of The University of Queensland. Adult, male SD rats were purchased from The University of Queensland Medical School Animal Breeding facility or from Australian Animal Resources (Melbourne, Australia). Rats were housed in a temperature-controlled room (21 ± 2°C) with a 12-h light/dark cycle, with food and water available ad libitum. Rats were given an acclimatization period of at least 4 days before surgery.

**Reagents and Materials.** Morphine sulfate ampoules (30 mg/ml) and chloramphenicol succinate vials (1.2 g) were purchased from the Royal Brisbane Hospital Pharmacy. Ketamine hydrochloride vials (100 mg/ml) were obtained from Parnell Laboratories Australia Pty Ltd (Sydney, Australia) and xylocaine hydrochloride (Ilium Xylazil-20) vials (20 mg/ml) were purchased from Troy Laboratories Pty Ltd (Sydney, Australia). Isoflurane (Forthane) was obtained from Abbott Australasia Pty Ltd (Sydney, Australia). Sodium benzylenicillin vials (600 mg) were purchased from CSL Ltd (Melbourne, Australia). Normal saline and lignocaine ampoules were obtained from Delta West Pty Ltd (Perth, Australia) and heparinized saline (50 U.I./5 ml) was purchased from Astra Pharmaceuticals Pty Ltd (Sydney, Australia).

Single lumen polyethylene tubing (i.d. 0.5 mm, o.d. 1.00 mm) was purchased from Auburn Plastics and Engineering Pty Ltd (Sydney, Australia). Sterile siliconized silk sutures (Dysilk) were obtained from Dynek Pty Ltd (Adelaide, South Australia) and Michel clips were purchased from Medical and Surgical Requisites Pty Ltd (Brisbane, Australia). Denture acrylic and denture monomer were purchased from Regional (Brisbane, Australia). Hamilton syringes were used for i.c.v. drug administration, and Graseby medical syringe drivers were used to administer infusions (Graseby Medical Ltd, Gold Coast, Australia).

### Cannula Preparation

Jugular vein and femoral artery cannulas were made from approximately 55- and 30-cm lengths of polyethylene tubing, respectively. Using heat shrink, silastic tubing (≈2 cm) was joined to one end of the jugular catheter for insertion into the vein. To facilitate femoral artery cannula insertion, the diameter of one end (≈1 cm) of the tubing was decreased to approximately half its original size by gentle stretching. Guide cannulas (i.c.v.) were cut from 21-gauge stainless steel needles to a length of 8 mm and filed to a 45° bevel. Cannula plugs were cut from 25-gauge needles to a length of 9 mm.

### Surgery. Jugular vein and/or femoral artery cannulation.

At the time of surgery, rats weighed 440 ± 40 g, mean ± S.E.). Femoral artery and/or jugular vein cannulation was performed while rats were anesthetized with 3% isoflurane: 97% O$_2$, inhalational anesthesia and was maintained using a calibrated Trilene vaporizer. Polyethylene cannulas (previously filled with heparinized saline) were implanted into the right common jugular vein for both acute and chronic i.v. morphine dosing. For rats in the chronic (48-h) studies, cannulas (previously filled with heparinized saline) were inserted approximately 3.5 to 4.5 cm into the femoral artery, such that the tip of the cannula was positioned well within the dorsal aorta but below the level of the renal veins (Waynforth and Flecknell, 1992). Cannulas were exteriorized by s.c. tunneling to an incision made in the interscapular area, and protected by a stainless steel spring, the base of which was positioned in a s.c. pocket between the scapulae. Incisions were closed with sterile silk sutures and for the femoral artery cannula, the incision site was reinforced with Michel clips. After surgery, rats were housed singly in metabolic cages and were allowed to recover postoperatively for a minimum of 1 h before more experimentation. Unlimited access to food and water was permitted during the recovery period.

### Implantation of i.c.v. guide cannulas. Rats (250 ± 12 g, mean ± S.E.) were anesthetized with a mixture of xylazine (8 mg/kg) and ketamine (80 mg/kg) administered by single i.p. injection while under light anesthesia (50% O$_2$/50% CO$_2$). This combination anesthetic produced deep and stable anesthesia for the duration of the surgical procedure. Lignocaine was administered as a topical local anesthetic to reduce postsurgical pain around the sutures. Stainless steel guide cannulas were implanted stereotaxically to a depth of 1 mm above the right lateral ventricle using the rat brain co-ordinates of Paxinos and Watson (1986; 0.8 mm posterior, 1.5 mm lateral, and 3.3 mm ventral relative to bregma). Guide cannulas were secured in position with dental cement and the scalp wound was sutured. Cannula plugs were kept in place except during drug injections. After surgery, rats received benzylpenicillin (30 mg i.m.) to prevent infection. Rats were allowed to recover for 5 days before femoral artery cannula insertion. Sham-operated rats received the same surgical incisions as the experimental rats and were sutured, but without cannula implantation.

### Drug Dosing Regimens.

Chloramphenicol solutions were prepared by reconstituting chloramphenicol succinate lyophilized powder with heparinized saline to a final concentration of 600 mg of chloramphenicol in a volume of 9 ml. Chloramphenicol solutions were refrigerated and kept for up to 30 days as they are stable at room temperature for this period of time (Trissel, 1992). Solutions of morphine for i.c.v. and i.v. administration were prepared from morphine sulfate ampoules and were diluted to the appropriate strength (calculated as morphine base) with sterile saline. Morphine solutions were frozen at −20°C until required. The concentration of morphine in each solution was verified using our HPLC method with electrochemical detection (Knight et al., 1994).

### Systemic dosing. Acute studies.

Two groups of rats (n = 8) received chloramphenicol succinate (200 mg/kg) or saline as a brief i.v. infusion (≈30 s) via the jugular vein cannula followed immediately by 0.2 ml of heparinized saline (50 U.I./5 ml) to ensure delivery of the full dose into the systemic circulation. Thirty minutes later, these rats received morphine (4.25 mg/kg, n = 5) via the jugular cannula followed by 0.2 ml of heparinized saline. Each group of control rats (n = 3) received the same volume of vehicle.

### Systemic dosing: Chronic studies.

Chloramphenicol succinate (100 mg) was administered as an i.v. bolus (over 30 s) followed 30 min later by an i.v. bolus of morphine (4.25 mg/kg) through the same jugular cannula. Chloramphenicol succinate (300 mg/24 h) and morphine (10 mg/24 h) were then infused chronically via the femoral artery and the jugular vein cannulas, respectively, at a flow rate of 4.5 ml/24 h. Control rats received the respective vehicle infusions at the same rate and in the same volume as the active drugs. The daily dose of morphine administered in these studies was determined in preliminary.
experiments to be the maximum survival dose tolerated by adult male SD rats coadministered chloramphenicol. Higher doses of morphine coadministered with chloramphenicol resulted in death, which appeared to be due to respiratory depression and/or neuroexcitation. The dose of chloramphenicol administered was based on the milligram per kilogram doses used for veterinary purposes in small companion animals.

Intracerebroventricular morphine administration. Groups of rats (n = 6) received i.a. chloramphenicol (100 mg bolus, then 300 mg/24 h) or saline for 48 h before i.c.v. administration of one of the following doses of morphine: 99, 117, 135, 153, 158, 162, and 171 nmol (2 μl), using an i.c.v. injection rate of 2 μl/min. Sterile normal saline (2 μl) was administered to control rats. Sham-operated rats (n = 4) received no drugs. After completion of the experiment, i.c.v. cannula placement was verified by i.c.v. injection of malachite green dye (1 μl) after decapitation and gross dissection of the brain. Diffusion of the dye throughout the ventricles and an absence of clumping in the periventricular tissue confirmed correct i.c.v. cannula placement.

Antinoceptive Testing. Antinoception was assessed using the hotplate latency test (Eddy and Leimbach, 1953) at 55 ± 0.5°C, a temperature at which both Aδ- and C-fibers are activated (Guyton, 1987). Individual rats were placed on the hotplate (model 39D; IITC Life Science, Woodland Hills, CA) and observed for the first behavioral signs of nociception (paw licks, jumping on the hotplate, vocalization, or escape attempts), whereas the timer was stopped and the rat was removed from the hotplate. A cut-off time of 30 s was used to minimize tissue damage to the rats’ paws. Predosing reaction times were typically 3 s and were the average of three readings taken approximately 5 min apart. After i.c.v. morphine (or saline) administration, antinoceptive testing was performed at the following times: 5, 10, 15, 30, 45, 60, 75, 90, 120, 150, and 180 min. For studies of the antinoceptive effects of i.v. morphine given acutely or chronically, antinoceptive testing was performed immediately before blood sample collection (see below) for the 3- and 48-h study periods, respectively.

Hotplate latency times were converted to %MPE using the following formula (Brady and Holtzmann, 1982):

\[
\%\text{MPE} = \frac{\text{Postdrug latency} - \text{Predrug latency}}{\text{Maximum latency (30 s)}} \times 100
\]

Blood Sample Collection. For the acute i.v. studies (3 h), blood samples (0.4 ml) were collected via the jugular cannula immediately before administration of chloramphenicol (or saline), and 30 min later before morphine or saline (control rats) administration. Blood samples were also collected at the following postdosing times: 5, 10, 15, 30, 45 min, 1.0, 1.5, 2, and 3 h. After centrifugation, serum was separated using polypropylene pipettes and frozen at −20°C until time of assay. Blood sample collection during the chronic (48-h) experiment was from the femoral artery rather than the jugular vein to preclude possible contamination of blood samples with the morphine infusion solution. Blood samples (0.55 ml) were collected predosing as described above for the acute studies and at the following times after initiation of the morphine infusion: 0.5, 1.0, 2, 3, 6, 12, 24, 30, 36, and 48 h. Fewer blood samples were collected from control rats not receiving morphine at the following times: 3, 6, 12, 24, 30, 36, and 48 h as preliminary studies showed that this did not significantly alter the baseline levels of antinoception observed throughout the experimental period.

Quantitation of Morphine and M3G in Rat Serum. Serum concentrations of morphine and M3G were quantified using our HPLC method with electrochemical detection (Wright et al., 1994). The lower limits of quantification for morphine and M3G were 2.3 and 5 ng injected on column, respectively. The recoveries of morphine and M3G from rat serum were 93.8 ± 6% (n = 9) and 93.1 ± 2.2% (n = 9), respectively (Wright et al., 1994). Standard curves comprising six to seven concentrations of morphine and M3G were chromatographed in random order with each batch of rat serum samples. Peak height ratios of either morphine or M3G relative to that of the internal standard were plotted against concentration. Regression analysis was used to produce standard curves, which were accepted if the correlation coefficients were ≥0.99. Additionally, control serum samples (‘seeds’ in two different concentrations (17.0 or 170 ng of morphine and 46.9 or 469 ng of M3G per sample) were included in each chromatographic run at a frequency of approximately one ‘seed’ per five samples. The %MPE values for the ‘low seeds’ for morphine and M3G were 8.8 and 12.5%, respectively, whereas for the ‘high seeds’ the respective c.v. values were 7.0 and 10.4% for morphine and M3G, respectively.

Pharmacokinetic Analysis. The morphine serum concentration-time data obtained from rats that received single doses of morphine (groups 1 and 2) were analyzed using an iterative parameter estimation algorithm (Marquardt, 1963) as implemented in the Stemkinetics pharmacokinetic modeling program (Smith and Smith, 1987). A polyexponential equation of the form:

\[
C = \sum_{i=1}^{n} A_i e^{-\beta_i t}
\]

was fitted to the data, where \(A_i\), \(\alpha_i\) = model parameters, \(C\) = serum drug concentration, \(t\) = time, \(n\), \(i\) = constants.

Areas under the serum morphine and M3G concentration-time curves (AUCs) were determined by trapezoidal integration to ‘t’ hours. For acute morphine dosing, AUC\(_t\) was calculated by dividing C, by the terminal elimination rate constant, β (Gibaldi, 1984). The terminal elimination half-life, \(t_{1/2}\), was calculated from the relationship \(t_{1/2} = 0.693/\beta\).

The total plasma clearance, CL, was determined from the relationship CL = FD/AUC, where D = dose, F = bioavailability (= 1 for i.v. dosing), and AUC = area under the serum drug concentration-time curve from \(t = 0\) to \(\infty\). The apparent volume of distribution, V, was determined from the relationship \(V = CL/\beta\).

Statistical Analyses. Comparisons between experimental groups for a range of pharmacokinetic parameters for morphine and M3G and for the area under the %MPE versus time curve (extent and duration of morphine antinociception; %MPE AUC) were performed using the Wilcoxon Rank-Sum test as implemented in the Stemstats statistical analysis package (Smith and Smith, 1987). The statistical significance criterion was \(P < 0.05\). Regression analysis was used to determine whether there was any significant correlation between mean levels of antinoception and the mean values of the serum molar concentration ratio, M3G/morphine, for each experimental group. For i.v. dosing, dose-response curves were produced by plotting either %MPE AUC or the %MPE value at the time of maximum response (45 min) versus the magnitude of the i.c.v. morphine dose. The ED\(_{50}\) values were estimated from the dose-response curves using GraphPad Prism. Comparisons between ED\(_{50}\) values for the two experimental groups were also performed using the Wilcoxon Rank-Sum test with a statistical significance criterion of \(P < 0.05\).

Results

Systemic coadministration of chloramphenicol with i.v. morphine increased both the extent and duration of morphine antinociception (%MPE AUC, Table 1), irrespective of whether these drugs were coadministered acutely (Fig. 1A) or chronically (Fig. 1B). However, pretreatment of rats with i.a. chloramphenicol for 48 h did not significantly alter the intrinsic antinoceptive effects of i.c.v. morphine (99–171 nmol; Fig. 1, C and D). Importantly, coadministration of the same dose of chloramphenicol with i.v. saline did not alter baseline levels of antinoception during the study period (Fig. 1F).

**TABLE 1**

<table>
<thead>
<tr>
<th>Experimental Group</th>
<th>Mean (±S.E.) %MPE AUC after acute and chronic parenteral coadministration of chloramphenicol and morphine to adult male SD rats</th>
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<tbody>
<tr>
<td>Acute i.v. Morphine</td>
<td></td>
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<tr>
<td>1 (+ i.v. saline)</td>
<td>36.4 ± 9.9</td>
</tr>
<tr>
<td>2 (+ i.v. chloramphenicol)</td>
<td>197.9 ± 23.9</td>
</tr>
<tr>
<td>Chronic i.v. Morphine</td>
<td></td>
</tr>
<tr>
<td>A (+ i.a. saline)</td>
<td>243 ± 47</td>
</tr>
<tr>
<td>B (+ i.a. chloramphenicol)</td>
<td>1338 ± 244</td>
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CHLORAMPHENICOL INCREASES I.V. MORPHINE ANTINOCICEPTION

Fig. 1. A, mean (±S.E.) degree of antinociception versus time curve after single dose i.v. administration of chloramphenicol (200 mg/kg) + morphine (4.25 mg/kg) (○) (i) or saline + morphine (4.25 mg/kg) (■) (ii). B, mean (±S.E.) degree of antinociception versus time curve after chronic 48-h parenteral administration of chloramphenicol (300 mg/24 h) + morphine (10 mg/24 h) (○) (i) or saline + morphine (10 mg/24 h) (■) (ii). C, mean (±S.E.) degree of antinociception versus time curve after i.c.v. administration of morphine (162 nmol) to rats pretreated for 48 h with i.a. chloramphenicol (□) or saline (●). Levels of antinociception were not significantly (P > .05) altered by chloramphenicol pretreatment. D, mean (±S.E.) dose-response curves produced using the %MPE AUC data for i.c.v. morphine (99–171 nmol) administered to rats pretreated for 48 h with i.a. chloramphenicol (□) or saline (●). E, mean (±S.E.) dose-response curves produced using the peak levels of antinociception observed at 45 min after i.c.v. morphine (99–171 nmol) administration to rats pretreated for 48 h with i.a. chloramphenicol (□) or saline (●). F, mean (±S.E.) levels of antinociception observed in sham-operated (△) and control rats after i.c.v. administration of saline (2 μl) to rats pretreated for 48 h with i.a. chloramphenicol (□) or saline (●).
Intracerebroventricular Morphine: Antinociception and Dose-Response Curves. Irrespective of whether rats were pretreated with i.a. chloramphenicol or i.a. saline for 48 h before i.c.v. morphine (162 nmol) administration, the antinociceptive response comprised two distinct phases as expected based on our previous studies (Smith et al., 1990; Leow and Smith, 1994; Ross and Smith, 1997). The first phase of antinociception commenced at approximately 15 min, peaked at 30 to 45 min, and decreased at 60 min. The second phase of antinociception peaked at 90 min and decreased throughout the remainder of the 3-h observation period (Fig. 1C). Similar results were obtained for each of the other i.c.v. morphine doses administered. Visual inspection and statistical analysis of the dose-response curves (Fig. 1, D and E) show that there are no significant differences (P > .05) in the antinociceptive effects of i.c.v. morphine between rats that were pretreated with chloramphenicol and those that were not. Specifically, the mean (±S.E.) ED$_{50}$ for i.c.v. morphine (based on the %MPE AUC data) in the absence of chloramphenicol (157.4 ± 2.5 nmol) was not significantly different (P > .05) from that (156.8 ± 2.4 nmol) of rats pretreated with chloramphenicol. Similarly, the mean (±S.E.) ED$_{50}$ for i.c.v. morphine in the absence and presence of chloramphenicol (156.2 ± 2.4 and 156.0 ± 2.3 nmol, respectively) did not differ significantly (P > .05) when determined from the dose-response relationship derived from the peak %MPE values determined at 45 min postdosing (Fig. 1E).

Levels of antinociception observed in sham-operated rats and control rats that received i.c.v. saline after pretreatment with parenteral saline or chloramphenicol were not significantly different (P > .05) from baseline throughout the 3-h study period (Fig. 1F). Thus, parenteral chloramphenicol does not produce antinociception in rats and the experimental procedures themselves do not contribute to antinociception. Behaviorally, rats that received i.c.v. morphine were sedated and experienced urinary incontinence during the second phase of antinociception (75 min onwards). By contrast, rats that received i.c.v. saline together with parenteral chloramphenicol or saline were indistinguishable from sham-operated rats.

Systemic Morphine: Acute Studies. Antinociception. For rats administered i.v. morphine (4.25 mg/kg) alone (group 1, n = 5), high levels of antinociception (>60% MPE) were observed for the first 15 min (Fig. 1A). Thereafter, %MPE values decreased, reaching baseline (<10% MPE) at 60 min (Fig. 1A). By contrast, maximum antinociception (100% MPE) was observed for 45 min in rats coadministered chloramphenicol (200 mg/kg) with the same dose of morphine (group 2, n = 5). Levels of antinociception (%MPE values) decreased thereafter, but baseline levels were not reached by the end of the 3-h study period in four of the five rats in this group (Fig. 1A). The mean (±S.E.) %MPE AUC was approximately 5.5-fold greater for rats coadministered chloramphenicol and morphine (197.9 ± 23.9%MPE h) relative to that for rats that received the same dose of i.v. morphine alone (36.4 ± 9.9%MPE h, Table 1).

Levels of antinociception observed in control rats dosed with either vehicle (group 3, n = 3) or chloramphenicol (group 4, n = 4) were not significantly different from predosing baseline levels of antinociception throughout the 3-h experimental period (data not shown), indicating that neither chloramphenicol nor the experimental procedures evoked antinociception.

Serum concentrations of morphine. At 5 min postdosing in group 1 rats (morphine + saline), the mean (±S.E.) serum morphine concentration was 4.56 (± 0.93) μM (Fig. 2A), which decreased biexponentially with a mean (±S.E.) serum half-life (t$_{1/2}$) of 0.71 (± 0.10) h and a mean (±S.E.) CL of 2.22 ± 0.25 liters·h$^{-1}$ (Table 2). The respective mean (±S.E.) volume of distribution (V) for morphine was 2.35 (± 0.57) liters and the mean (±S.E.) morphine AUC (morphine AUC) was 2.67 ± 0.34 μmol·liter$^{-1}$·h (Table 2). The mean (±S.E.) serum morphine concentration at 5 min postdosing (6.06 ± 0.30) μM in group 2 rats (chloramphenicol + morphine), was approximately 25% higher than the corresponding concentration in group 1 rats (Fig. 2A). The mean morphine concentration remained significantly higher (P < .05) in group 2 relative to group 1 rats for the first 2 h of the 3-h experimental period (Fig. 2A), such that the mean (±S.E.) morphine AUC (4.67 ± 0.55 μmol·liter$^{-1}$·h) was approximately 75% larger than that for group 1 rats. In group 2
Chloramphenicol significantly increased i.v. morphine antinociception.

### Systemic Morphine: Chronic Studies. Antinociception.

Rats administered i.v. morphine chronically (4.25 mg/kg bolus followed by 10 mg/24 h infusion, group A, \( n = 10 \)) exhibited high levels of antinociception (\( >60\% \text{MPE} \)) for the first 0.5 h of the 48-h infusion period. Thereafter, levels of antinociception decreased, reaching baseline (\(<10\% \text{MPE} \)) at 3 h and remaining at baseline until completion of the 48-h study period (Fig. 1B). By contrast, high levels of antinociception (\( >60\% \text{MPE} \)) were observed in rats coadministered chloramphenicol and morphine (group B, \( n = 10 \)) for a significantly longer period (\( P < .05 \)) time (6 h) than in rats dosed with morphine alone (0.5 h). Levels of antinociception decreased to baseline at 30 h and remained at baseline until the end of the study period (Fig. 1B). For the first 24 h of the infusion period, the mean (\( \pm \text{S.E.} \)) %MPE AUC was \( \approx 5.5\)-fold larger (\( P < .05 \)) for rats in group B (1338 \( \pm \) 244\%MPE h) relative to that for rats in group A (243 \( \pm \) 47\%MPE h) (Table 1).

### Serum morphine concentrations.

For rats administered morphine alone (group A), the mean (\( \pm \text{S.E.} \)) serum morphine concentration was 2.92 (\( \pm \) 0.29) \( \mu \)M at 15 min, which decreased to 2.01 (\( \pm \) 1.02) \( \mu \)M at 2 h. At 6 and 12 h, the mean (\( \pm \text{S.E.} \)) morphine concentrations were 0.40 (\( \pm \) 0.07) \( \mu \)M and 0.44 (\( \pm \) 0.08) \( \mu \)M, respectively (Fig. 3A). Thereafter, the mean (\( \pm \text{S.E.} \)) serum morphine concentration appeared to undergo enterohepatic recirculation, with a second absorbptive peak of morphine (1.06 \( \pm \) 0.45 \( \mu \)M) occurring at 30 h, which decreased to 0.54 (\( \pm \) 0.06) \( \mu \)M at 48 h.

For rats coadministered chloramphenicol and morphine (group B), the mean (\( \pm \text{S.E.} \)) serum morphine concentration was 2.22 (\( \pm \) 0.78) \( \mu \)M at 15 min, which decreased to 1.39 (\( \pm \) 0.13) \( \mu \)M at 2 h and to 0.69

### Table 2

<table>
<thead>
<tr>
<th>Pharmacokinetic Parameter</th>
<th>Morphine</th>
<th>M3G</th>
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<tr>
<td></td>
<td>Group 1 (+ Saline)</td>
<td>Group 2 (+ Chlor)</td>
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<tr>
<td>Acute i.v. Morphine (4.25 mg/kg)</td>
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<td></td>
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<tr>
<td>( t_{1/2} ) (h)</td>
<td>0.71 ( \pm ) 0.10</td>
<td>0.81 ( \pm ) 0.11</td>
</tr>
<tr>
<td>AUC(0→t) (( \mu )mol · liter(^{-1} ) · h(^{-1} ))</td>
<td>2.67 ( \pm ) 0.34</td>
<td>4.67 ( \pm ) 0.55( \ast )</td>
</tr>
<tr>
<td>CL (liter · min(^{-1} ))</td>
<td>2.22 ( \pm ) 0.25</td>
<td>1.38 ( \pm ) 0.12( \ast )</td>
</tr>
<tr>
<td>V (liter)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chronic i.v. Morphine (10 mg/24 h)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>AUC(0→24 h) (( \mu )mol · liter(^{-1} ) · h(^{-1} ))</td>
<td>18.3 ( \pm ) 4.5</td>
<td>23.0 ( \pm ) 3.1</td>
</tr>
<tr>
<td>AUC(0→48 h) (( \mu )mol · liter(^{-1} ) · h(^{-1} ))</td>
<td>35.9 ( \pm ) 8.7</td>
<td>36.9 ( \pm ) 4.7</td>
</tr>
</tbody>
</table>

* Groups 1 and 2 significantly different (\( P < .05 \)).

### RESULTS

**Pharmacokinetic Parameters:**

After i.v. administration to rats in group 1, morphine was metabolized rapidly to M3G, as indicated by the relatively high mean (\( \pm \text{S.E.} \)) serum M3G concentration (1.28 \( \pm \) 0.08) at 180 min. For rats in both groups, good inverse exponential correlations were obtained between the mean (\( \pm \text{S.E.} \)) levels of antinociception observed and the mean (\( \pm \text{S.E.} \)) values of the serum molar concentration ratio, M3G/morphine, (group 1: \( r^2 = 0.946, P < .001 \); group 2: \( r^2 = 0.849, P < .001 \)), although these correlations were different between the two groups (Fig. 2C).

**Behavioral effects:** Rats that received morphine (4.25 mg/kg) alone (group 1) were markedly sedated for the first 10 to 15 min, but they recovered by 30 to 45 min postdosing. By 90 to 120 min, all group 1 rats appeared to be fully aware of their surroundings and exhibited normal eating, grooming, and exploring behaviors. By contrast, a significantly (\( P < .05 \)) longer period of marked sedation (45–120 min) was observed in rats (group 2) coadministered chloramphenicol (200 mg/kg) and morphine (4.25 mg/kg), and considerable interindividual variability was noted. Control rats dosed with saline or chloramphenicol plus saline exhibited similar eating, drinking, grooming, and exploring behaviors to untreated rats, further emphasizing chloramphenicol’s lack of central nervous system (CNS) effects in this study.
Serum M3G concentrations. The mean (± S.E.) serum M3G concentration for rats in group A (morphine alone) peaked at 1.77 (±0.25) μM at 0.5 h, decreasing thereafter to 0.58 (±0.13) μM at 6 h (Fig. 3B). A second absorptive peak was observed in the mean (± S.E.) serum M3G concentration-time curve at the same time (30 h) as that observed for morphine. In group B rats, the mean (± S.E.) serum M3G concentration increased from 1.06 (±0.06) μM at 15 min to 1.31 (±0.15) μM at 6 h (Fig. 3B), thereafter decreasing to a steady-state concentration of approximately 0.80 μM at 24 h. The mean (± S.E.) serum M3G AUC (22.8 ± 4.1 μmol · liter⁻¹ · h) for rats coadministered chloramphenicol and morphine (group B) was significantly (P < .05) larger (∼40%), not smaller, as one might have predicted, than the respective value for group A rats (15.9 ± 3.5 μmol · liter⁻¹ · h) for the first 24 h of the infusion period. However, the M3G AUC values for the entire 48-h study period did not differ significantly (P > .05) between rats in groups A and B (Table 2), as was observed for the morphine AUC values.

Serum samples collected from control rats (administered saline and/or chloramphenicol) did not contain compounds that cochromatographed with either morphine or the internal standard. The plasma creatinine concentrations determined in the predosing and 48-h serum samples collected from rats in this study were within the normal range (data not shown), indicating that rats had normal renal function during the 48-h experimental period.

Serum molar concentration ratio, M3G/morphine. In rats infused chronically with morphine alone (group A), the mean (± S.E.) value of the serum molar concentration ratio, M3G/morphine, was 0.63 (±0.14) at 15 min (Fig. 3C), which increased to a steady-state value of ∼1.5 at 6 h. By contrast, the mean (± S.E.) values of the M3G/morphine ratio in rats coadministered chloramphenicol (group B), increased relatively linearly from 0.67 (±0.30) at 15 min to 2.49 (±0.65) at 48 h.

In both group A and group B rats, the mean (± S.E.) %MPE values were highly inversely correlated with the mean (± S.E.) values of the serum molar concentration ratio, M3G/morphine (group A: r² = 0.955, P < .0001; group B: r² = 0.775, P < .0005), but these respective inverse correlations were different between groups A and B (Fig. 3C). The inverse correlation observed in group A rats was similar to that observed previously in our laboratory (Smith and Smith, 1995) for rats that received chronic i.v. morphine infusions (30–80 mg/24 h) such that %MPE ≥ 70% corresponded with M3G/morphine ratio values <0.5. Levels of antinociception decreased with increasing values of the M3G/morphine ratio such that for values <1.1, the corresponding levels of antinociception observed were not significantly different (P > .05) from predosing baseline values or from levels of antinociception obtained in control rats. The inverse correlation between mean levels of antinociception (%MPE values) and the mean serum M3G/morphine molar concentration ratio in group B rats, appeared to be displaced to the right by a factor of ∼1.5 relative to that determined in rats that received chronic i.v. infusions of morphine alone (group A).

Behavioral effects. Rats that were infused chronically with i.v. morphine alone (group A) were markedly sedated for the first 0.5 to 2.0 h of the 48-h experimental period, during which the rats were unresponsive to the non-noxious stimuli of sound and touch. By 3 to 6 h, rats were awake and responsive to sound and touch. From 12 h onwards, all rats were fully awake with normal eating, grooming, and exploring behaviors. Rats coadministered chloramphenicol and morphine (group B) remained sedated for significantly (P < .05) longer (3–12 h) than the corresponding rats in group A. Rats coadministered chloramphenicol and morphine recovered slowly and remained quiet and inactive for the remainder of the 48-h experimental period. In

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**Fig. 3.** A, mean (±S.E.) serum morphine concentration versus time curves for rats coadministered i.a. chloramphenicol (300 mg/24 h) + i.v. morphine (10 mg/24 h) (○) or i.a. saline + i.v. morphine (10 mg/24 h) (■), for 48 h. B, mean (±S.E.) serum M3G concentration versus time curves for rats coadministered i.a. chloramphenicol (300 mg/24 h) + i.v. morphine (10 mg/24 h) (○) or i.a. saline + i.v. morphine (10 mg/24 h) (■), for 48 h. C, significant inverse correlations between mean (±S.E.) levels of antinociception and the mean (±S.E.) values of the serum molar concentration ratio, M3G/morphine, in rats dosed chronically for 48 h with i.v. chloramphenicol (300 mg/24 h) + morphine (10 mg/24 h) (○) or i.a. saline + morphine (10 mg/24 h) (■).
addition to marked sedation, one-third of rats in this group (5 of 15) appeared to experience respiratory depression and/or neuroexcitation, resulting in death. Additionally, it was noted that for the duration of these experiments, rats given morphine (groups A and B) were not as active at night as control rats (administered saline and/or chloramphenicol), even when apparently fully tolerant to the antinociceptive effects of morphine.

Control rats dosed chronically with saline (n = 6) or chloramphenicol plus saline (n = 3) exhibited behavior indistinguishable from that of untreated rats. Specifically no antinociception, respiratory depression, sedation, or excitatory behaviors were noted in either group. All rats exhibited normal eating, grooming, and exploring behavior and were active at night, consistent with their nocturnal behavior.

Discussion

Parenteral coadministration of chloramphenicol and morphine in adult male SD rats increased morphine antinociception by ≈5.5-fold when assessed using the hotplate latency test, irrespective of whether the drugs were administered acutely or chronically (Fig. 1, A and B). However, pretreatment of rats with chloramphenicol for 48 h before i.c.v. morphine administration did not significantly alter the levels of antinociception observed (ED_{50} ≈ 156 nmol in the presence or absence of chloramphenicol) (Fig. 1, C–E). Taken together, these data strongly suggest that parenteral chloramphenicol augments the antinociceptive effects of i.v. morphine via a mechanism that does not directly involve supraspinal opioid receptors in the rat CNS. Although both acute and chronic coinfusion of chloramphenicol and morphine resulted in a similar 5.5-fold increase in the %MPE AUC values of i.v. morphine, the corresponding serum morphine AUC values were altered to different extents, ≈75% increase and no significant change, respectively, relative to the same doses of acutely or chronically administered i.v. morphine alone (Table 2). Clearly, these data indicate that factors other than just the serum morphine concentrations contribute significantly to the levels of antinociception observed.

Quantification of the serum M3G concentrations and the corresponding AUC values in rats coadministered chloramphenicol revealed unexpectedly that the M3G AUC values were not lower but were significantly (P < .05) higher (33 and 44% for the acute and chronic studies, respectively) than the respective M3G AUC values determined in rats dosed with morphine alone (Tables 1 and 2; Figs. 2B and 3B). However, as chloramphenicol was administered to rats in a greater than 50-fold molar excess relative to morphine, the much larger amount of chloramphenicol glucuronide formed relative to M3G may have significantly inhibited the biliary secretion of M3G. Evans and Shanahan (1995) showed, using isolated perfused adult male SD rat livers, that 30% of the morphine dose metabolized to M3G was secreted into the biliary bile. Thus, if chloramphenicol glucuronide competitively inhibited the biliary secretion of M3G, this would have produced higher than expected M3G serum concentrations, as was found in both our acute and chronic studies, and decreased subsequent enterohpatic recycling of morphine. Furthermore, the apparent volume of distribution of morphine would have been decreased in accordance with our findings of an ≈35% decrease in the volume of distribution of morphine from 2.35 (±0.57) to 1.53 (±0.10) liters (Table 2) in rats coadministered chloramphenicol and morphine acutely. Using simulated data (Table 3), it is clear that a significant reduction in the biliary secretion of M3G and hence its volume of distribution would effectively ‘mask’ an inhibitory effect of chloramphenicol on the metabolism of morphine to M3G in rats coadministered chloramphenicol and morphine (group B) compared with rats dosed with morphine alone (group A).

Although the results of this study provide some evidence to support the proposal that ‘coadministration of chloramphenicol plus morphine results in in vivo inhibition of the glucuronidation of morphine to M3G’, this metabolic inhibition was incomplete. Such a finding is not unexpected, as in vitro studies using liver microsomes indicate that dual isoforms of uridine diphosphoglucuronosyltransferase are involved in the metabolism of morphine to M3G and that chloramphenicol competitively inhibits the low-affinity, high-capacity uridine diphosphoglucuronosyltransferase isoenzyme by ≈50% whereas it inhibits the high-capacity, low-affinity isoenzyme by only ≈10% (Miners et al., 1988; Coughtrie et al., 1989).

Our previous studies (Smith and Smith, 1995) and those of Barjavel et al. (1995) have shown a similar highly significant inverse correlation (r > 0.9) between mean levels of antinociception achieved and the mean values of the molar concentration ratio, M3G/morphine, in the plasma and cortical extracellular fluid, respectively, for rats dosed with parenteral morphine. In the studies described herein, a similar highly significant inverse correlation between mean levels of antinociception and mean values of the serum molar concentration ratio, M3G/morphine, was observed in rats dosed either acutely or chronically with morphine alone (Figs. 2C and 3C). However, for rats coadministered chloramphenicol and morphine (groups 2 and B), this inverse correlation appeared to be displaced to the right by a factor of ≈1.5 (Figs. 2C and 3C) relative to that observed for rats dosed with morphine alone (groups 1 and A). Although the reason for this difference isn’t clear, it is possible that inhibition of the glucuronidation of morphine to M3G by chloramphenicol in adult male SD rats may have resulted in a compensatory increase in the testosterone-dependent N-demethylation of morphine to normorphine (a less potent (≈30%) μ-opioid agonist (Miners et al., 1988; Blanck et al., 1990; Yue et al., 1990; Anandatheerthavarada and Ravindranath, 1991; Evans and Shanahan, 1995), resulting in higher serum and cerebrospinal fluid concentrations of this metabolite than in rats dosed with morphine alone. As the N-demethylation of morphine to normorphine reportedly accounts for ≈20% of the morphine dose in adult male SD rats (Evans and Shanahan, 1995), increased metabolism of morphine by this pathway may have pharmacodynamic consequences. Additional studies are required to investigate this possibility.

Previous studies have shown that the time required for the development of antinociceptive tolerance to morphine in rats is inversely correlated with the magnitude of the daily morphine dose (30–80 mg morphine sulfate/24 h) (Smith and Smith, 1995). Consistent with our previous findings, antinociceptive tolerance to the smaller daily dose of morphine administered to rats in this study (10 mg/24 h morphine base = 11.8 mg/24 h morphine sulfate) was complete by
12 h compared with 72 h for rats that received 30 mg/24 h of morphine sulfate in our previous study (Smith and Smith, 1995). However, it is possible that the shorter time to develop antinociceptive tolerance to the smaller dose of morphine (11.8 mg/24 h), may have been exaggerated by the different methods of antinociceptive testing used (tail-flick method in previous study versus hotplate method in this study).

The mean ED50 estimated for i.c.v. morphine (∼156 nmol) from the hotplate latency data in this study, was approximately 4- to 5-fold higher than that reported previously (∼33 nmol) for i.c.v. morphine using the tail-flick test (Nakata et al., 1990; Leow and Smith, 1994). This test-dependent difference in the apparent potency of i.c.v. morphine is consistent with our recent report that the tail-flick test is more sensitive than the hotplate test for detection of antinociception after parenteral morphine administration (South and Smith, 1998). The tail-flick test reportedly measures the latency of the spinal mediated reflexive withdrawal of the rat’s tail from the noxious radiant heat, a process that is influenced by the activity of supraspinal structures (Ramabadr and Bansinath, 1986). By contrast, the hotplate test is thought to involve supraspinal structures predominantly, as it requires an intact CNS (Pastoriza et al., 1996). Thus, our i.c.v. data indicate that at low doses, the antinociceptive effects of i.c.v. morphine are strongly influenced by spinal mechanisms. Although the i.c.v. doses of morphine required to elicit significant antinociception using the hotplate test were higher (>100 nmol) than those required to elicit maximum antinociception using the tail-flick test (∼80 nmol), the biphasic nature of the %MPE versus time curve was independent of the antinociceptive test used. Additionally, the duration of each antinociceptive phase was not significantly different between the hotplate (this study) and the tail-flick latency tests (Leow and Smith, 1994; Ross and Smith, 1997).

It should also be noted that parenteral coadministration of chloramphenicol and morphine not only increased morphine antinociception but it also significantly increased (P < .05) the duration of sedative effects of morphine, and resulted in respiratory depression and/or neuroexcitation, leading to the death of some rats. However, chloramphenicol’s attenuation of the pharmacological actions of morphine was not due to any direct antinociceptive or other effects of chloramphenicol, as rats dosed with chloramphenicol plus saline achieved only baseline levels of antinociception, were not sedated, and did not exhibit signs of respiratory depression or neuroexcitation. Moreover, control rats dosed with chloramphenicol were behaviorally indistinguishable from both untreated rats and rats dosed with vehicle alone.

In summary, coadministration of parenteral chloramphenicol with i.v. but not i.c.v. morphine resulted in a marked increase (5.5-fold) in the extent and duration of morphine antinociception, assessed using the hotplate test. These findings indicate that the marked enhancement of the antinociceptive effects of i.v. morphine by parenteral chloramphenicol is not mediated by a direct action of chloramphenicol at supraspinal opioid receptors in the rat CNS. All rats that received morphine by chronic i.v. infusion became tolerant to the antinociceptive effects of morphine, but tolerance developed more slowly in rats coadministered chloramphenicol. Our data suggest that chloramphenicol competitively inhibits the in vivo glucuronidation of morphine to its putative antinociceptive metabolite, M3G, with a possible compensatory increase in the N-demethylation of morphine to normorphine. Chloramphenicol also appears to significantly inhibit the hiliar secretion of M3G, thereby increasing rather than decreasing the corresponding serum M3G concentrations. Clearly, additional studies using brain microdialysis sampling in rats are required to determine whether chloramphenicol also influences the kinetics of the passage of morphine and/or M3G across the blood-brain barrier.