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# Interactions of Ethanol and Methadone

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by

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ABSTRACT

INTERACTIONS OF ETHANOL AND METHADONE

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The effects of ethanol administration on the antinociceptive activity, lethal properties and brain concentration of methadone, were investigated. The effect of ethanol on the antinociceptive activity of methadone was assessed by the hot-plate and tail-flick tests. Concentrations of methadone in the brain were determined by the use of $^{3}H$-methadone as well as by gas liquid chromatographic analysis. The study showed that moderate doses of ethanol did not alter tail-flick or hot-plate response by themselves. However, when combined with methadone, ethanol produced a significant increase in the antinociceptive effectiveness of methadone as measured by both a decrease in the $ED_{50}$ of methadone and by an increased intensity and prolonged duration of methadone antinociception. Ethanol increased the antinociceptive activity of methadone in both naive and methadone-tolerant mice. This increased activity was not due to simple addition of subthreshold effects of ethanol nor was it due to an ethanol-mediated increase in whole brain concentrations of methadone. It is hypothesized that the increased antinociceptive activity was the result of an ethanol-mediated increase in central nervous system sensitivity to the antinociceptive activity of methadone.

Ethanol pretreatment produced significantly lower brain concentrations of methadone compared to controls when methadone was administered subcutaneously. When both drugs were administered orally, ethanol administration resulted in brain concentrations of methadone initially less than control and at later times greater than control. In both ethanol and water-pretreated mice there was an excellent correlation between the whole brain concentration of methadone and antinociceptive effect, but the antinociceptive effect at any brain concentration of methadone was greater in ethanol-pretreated mice. Although ethanol produced significant alterations in the brain concentration of methadone, the brain concentration of ethanol was generally not altered by methadone administration. Investigations of the excretion of methadone and its metabolites and the half-life of methadone in the brain failed to reveal any significant ethanol-induced alterations.

A dose of ethanol which increased the antinociceptive activity of methadone did not alter the oral or subcutaneous $LD_{50}$ of methadone, although mice that died as a result of ethanol and methadone administration died at lower whole brain concentrations of methadone than those that died as a result of methadone alone. The $LD_{50}$ of ethanol was significantly decreased in mice maintained on a methadone dose of 100 mg/kg/day.

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# INTRODUCTION

## A) Methadone

Methadone exhibits a spectrum of pharmacological effects very similar to that of morphine (28) in that both drugs produce analgesia, respiratory depression and hypothermia. Soon after its discovery it was known that methadone could substitute for morphine in addicts and that similar to morphine, continued use of methadone produces tolerance and physical addiction in man (74), although advantage of this observation was not undertaken on a large scale until recently.

Like other narcotics, the analgesic effect of methadone is stereoselective. The l-isomer is approximately 20 times as effective an analgesic as the d-isomer following subcutaneous administration (146). The affinity of l-methadone for opiate receptors in brain homogenates is approximately 10 times that of the d-isomer but only one-fourth that of morphine (119). Although intrinsically less effective than morphine, the analgesic activity of methadone is enhanced by its high lipid solubility as evidenced by an oil/water distribution coefficient almost 1,000 times that of most other narcotics (80). This high lipid solubility accounts for two important properties of methadone. First, as shown by bolus injection of labeled drugs into the common carotid of the rat, the penetration of methadone through the blood-brain barrier is much greater than morphine and is almost as great as the uptake of heroin (115). In addition, methadone is very effective when given orally as evidenced by the fact that the $ED_{50}$ when given orally is only 8.5 times the $ED_{50}$ when given subcutaneously (146).

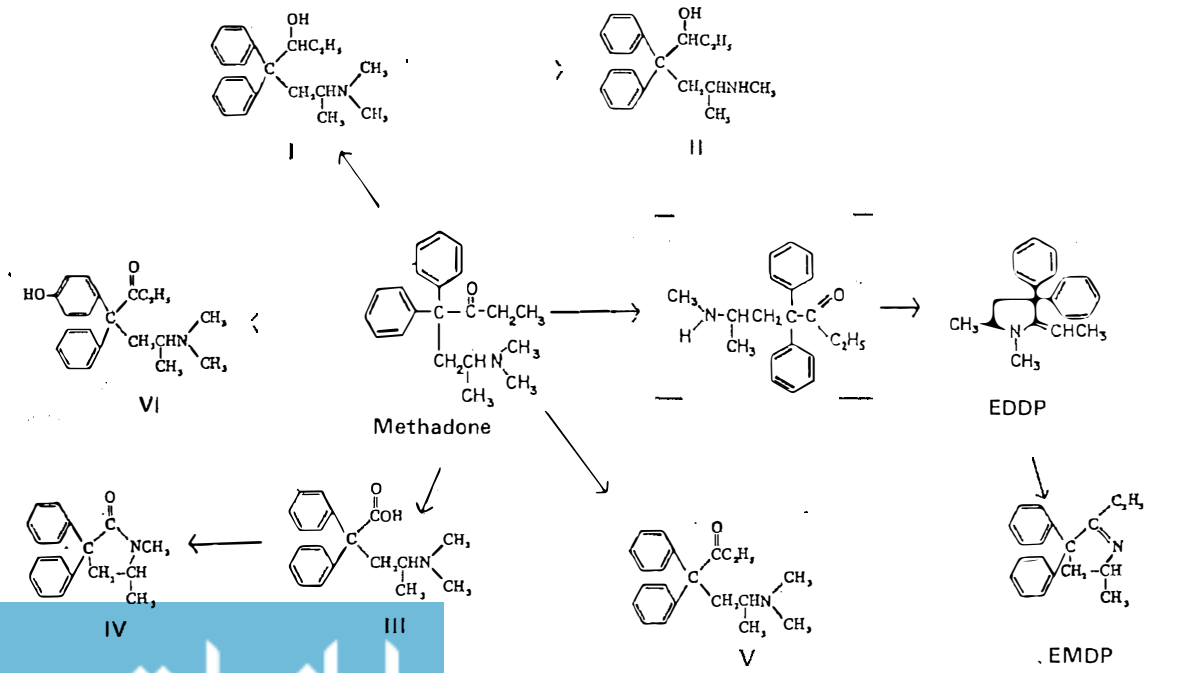
The structures of known metabolites of methadone in humans and rats are presented in figure 1. The major metabolic pathway for methadone is N-demethylation and subsequent non-enzymatic cyclization to 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP) and then further N-demethylation to 2-ethyl-5-methyl-3,3-diphenyl-1-pyrroline (EMDP) (124). Additional metabolites such as methadol (I), N-desmethyl methadol (II), 1,5-dimethyl-3,3-diphenyl-2-pyrrolidone (IV) as well as the conjugated hydroxy derivatives of methadone (VI), EDDP and EMDP have been isolated from human urine by various workers, most recently by Anggard et al. (5). Beckett (17) also has proposed the formation of methadone N-oxide (V), although other workers have not been able to substantiate this finding. The only metabolites which possess antinociceptive activity are the methadol and N-desmethylmethadol derived from d-methadone. The corresponding metabolites of l-methadone are almost inactive as analgesics (152).

Misra and Mule (107) have proposed that l-methadone but not d-methadone forms a methadone-derived compound which is tightly bound to brain tissue and is responsible for the differences in activity of the isomers and for the development of tolerance. Although there has been no direct evidence to refute this possibility, Sullivan et al. (151) reexamined the metabolism of both isomers and did not find any evidence to support this concept although they were looking only at metabolites excreted in the rat bile and not at metabolites in the brain. They suggest that the "tightly bound" radioactivity found by Misra and Mule is due to exchange or metabolism of their tritium label.

Although methadone gains access to the central nervous system (CNS)

FIGURE 1

METABOLISM OF METHADONE




Metabolites are identified in the text

to a greater degree than some narcotics, only a small portion of the total dose reaches the brain. Studies of the distribution of methadone in man (128), rat (94) and mouse (141) all show that the plasma and brain concentrations of methadone are very low in comparison to the levels found in the lung, liver, spleen and kidney. In addition to rapid passage of methadone from the general circulation to tissue sites, methadone has been found to be extensively bound to human plasma proteins (116). The high concentration of methadone in tissues may contribute to its long half-life in man by serving as a depot for the drug.

In non-tolerant individuals Verebely et al. (164) found a biexponential decay of plasma-methadone levels. The first phase had a half-life of 14 hours and the second phase had a half-life of 53 hours. As the patients became tolerant and the methadone dose was increased, the plasma half-life became mono-exponential with a half-life of 22 hours. In their study the percentage of the daily dose recovered in 24 hour urine and feces as methadone and EDDP was 19.2% and 3% respectively which increased to 42% and 20% respectively after 26 days of treatment. During the course of this study the daily dose of methadone was increased from 15 mg/day to 80 mg/day. The predominate product in the feces was EDDP while urine contained both methadone and EDDP. The urinary excretion of EDDP increased more rapidly than the excretion of methadone during treatment which, coupled with the shortened overall plasma half-life, was interpreted by the authors as indicating an induction of methadone metabolism.

The qualitative metabolism of methadone in rats appears to be the

same as in humans (108, 151). In rats with biliary fistulas, Baselt and Casarett (10) found that 54% of a subcutaneous dose of methadone was excreted via the bile as EDDP while only 4.2% and 6.0% was excreted in urine as EDDP and methadone, respectively. These authors found very little methadone or EDMP in bile but did find a water-soluble metabolite which accounted for 30% of the dose. They later (9) identified this metabolite as a conjugate of hydroxy-EMDP. By examining the partition coefficients of methadone, EDDP and EMDP, they found that EMDP is a somewhat unusual metabolite in that it is less polar than the parent methadone, and thus requires hydroxylation and conjugation in order to be polar enough to be excreted. Since it has been shown that methadone and its metabolites in bile are not significantly reabsorbed from the intestine (160), the high concentration of methadone metabolites in bile would indicate this to be a major pathway of excretion of methadone in intact rats.

Misra et al. (108) have shown that the half-life of methadone in rat brain after a subcutaneous dose is approximately 2.4 hours. Administration of methadone 10 mg/kg/day subcutaneously for 6 weeks shortened the half-life to 1.5 hours. This apparent demonstration of the ability of methadone to induce its own metabolism in vivo is supported by several studies (103, 148) where oral administration of methadone at doses in the range of 50 mg/kg/day produced a significant increase in the rate of methadone metabolism in vitro in the supernatant fraction of liver. These studies also tend to support the suggested increases in methadone metabolism seen in methadone maintenance patients.

The controlled, long-term administration of methadone for the purpose

of treating heroin addiction was begun by Dole and Nyswander in 1964 (44). Their approach was to administer gradually increasing doses of methadone until patients were receiving 80-120 mg/day. This treatment was designed to induce a high degree of tolerance to narcotics so that the addict would not feel the need for, nor derive any euphoric effect from a "normal" injection of an illicit narcotic. During prolonged use of these high doses of methadone, tolerance to almost all the narcotic effects is dramatic, although constipation and sweating often remain a problem. Medical examination of patients who have been in methadone maintenance programs for several years failed to reveal any significant alterations in the general health of the patients during the course of treatment (83).

The use of methadone maintenance unquestionably decreases the use of heroin. Even in the absence of counseling and additional programs aimed at rehabilitation, the simple removal of the constant need for the addict to find his next fix is a significant step in the alteration of the addict's life-style which should improve his chances to avoid future drug use. More recent programs have placed an increased emphasis on vocational, social and educational services in addition to simple dispensing of methadone to facilitate the reentry of the methadone maintenance patient into the mainstream of society (4). Although the substitution of methadone for heroin works well, the results of long-term followup of patients once they leave treatment are less encouraging. Even the original proponents of the treatment seem fairly pessimistic about the long-term "cure" (45). They cite statistics which show that of 204 patients who left treatment, almost half had resumed

use of illicit opiates. The remainder had become alcoholics, addicted to other drugs, been arrested or died. Only 22 of the 204 could be classified as in satisfactory status. Thus, while methadone maintenance provides the addict the opportunity to refrain from herion, the beneficial effect may last only as long as methadone maintenance is continued. Despite the poor prognosis after leaving, the benefits to the individual and society during treatment apparently justify the continuation of methadone maintenance, as evidenced by the growth in the number of people currently under treatment. From the initial pilot program in 1964 there were an estimated 80,000 people in methadone maintenance during 1975 (57).

This popularity has generated much interest in the relationship of dose to plasma level to the incidence of symptoms during long-term administration of methadone. It is clear from several studies that different patients maintained on the same dose of methadone exhibit a large variation in plasma methadone values at any given time (70, 164). This is probably a reflection of the fact that even in the same patient on the same dose there are dramatic (sometimes almost two-fold) alterations in the plasma-methadone level from week to week, although these alterations in plasma level are only rarely correlated with patient complaints (40). Goldstein (58) has shown that the use of 160 mg/day vs 80 mg/day does not generally produce a better treatment record when evaluated on the basis of clinic attendance, use of illicit opiates, or side effects reported. In addition, decreases of dose at the rate of 5 mg/week are generally not detected by the patient, although doses below 50 mg/day are often not satisfactory (57).

These observations indicate that above a certain level (e.g. 50 mg/day) the absolute level of methadone is not important in blockade of heroin use. The maintenance patient becomes tolerant to the daily dose, and gradual changes in plasma levels, whether due to intentional dose alteration or alterations in absorption, distribution, metabolism or excretion of the dose, are not detected by the patient. However, as also pointed out by Goldstein (58), rapid alterations in dose will be detected if they alter the amount of methadone available more rapidly than the level of narcotic tolerance is altered.

B) Ethanol

Ethanol passes rapidly across all mucosal membranes and thus is well absorbed from most routes of administration (167). Once absorbed, ethanol rapidly distributes to all tissues, the rate of equilibration with blood being largely a function of the degree of vascularization and rate of blood flow. Equilibration of ethanol with the brain, for example, is very rapid as evidenced by a single pass extraction ratio of 93% (40). The equilibrium distribution of ethanol is fairly uniform and generally follows the water content of the tissue. Thus, brain and liver ethanol concentrations are approximately equal and only slightly lower than blood ethanol concentrations (77), a distribution markedly different from most other drugs.

The major route of elimination of ethanol is by metabolism. Elimination of ethanol by excretion generally accounts for less than 10% of the total dose administered. The major, if not the only, enzyme responsible for the metabolism of ethanol in vivo is alcohol dehydrogenase

(ADH) (105). This enzyme reaches its maximum velocity at very low concentrations of ethanol and thus the metabolism of ethanol follows zero-order kinetics until blood-ethanol concentration falls to approximately 10 mg/100 ml (77). The ADH-mediated oxidation of ethanol to acetaldehyde results in reduction of NAD to NADH and it is the availability of NAD which limits the rate of ADH (165). The acetaldehyde formed is rapidly oxidized to acetate again with the reduction of NAD to NADH. Since the oxidation of acetaldehyde is faster than the rate of ethanol metabolism, acetaldehyde concentrations remain low and approximately constant irrespective of the concentration of ethanol (123).

In addition to ADH there are two other enzyme systems which have been proposed to play a role in the metabolism of ethanol in vitro. These are catalase in the presence of a hydrogen peroxide generating system (81), and the NADPH-dependent microsomal ethanol oxidizing system (MEOS) advocated by Lieber and coworkers (88). Although the in vivo rate of hydrogen peroxide production is too low to permit a significant contribution of catalase to the metabolism of ethanol in vivo, it has been proposed that the observed microsomal metabolism of ethanol is due to contamination by catalase (127). Whatever the exact nature of the microsomal metabolism of ethanol, its contribution to the metabolism of ethanol in vivo is probably small since pyrazole, which inhibits the activity of ADH in vitro, has little effect on MEOS, and does not alter catalase activity, is able to inhibit as much as 90% of the metabolism of ethanol in vivo (105).

Prolonged feeding of ethanol results in a metabolic tolerance which has been proposed to be a result of an induction of MEOS (87). However,

chronic ethanol feeding also increases the activity of the sodium-potassium activated ATPase which would increase the concentration of ADP (159). This would stimulate mitochondrial reoxidation of NADH and could also account for increased ethanol metabolism by way of ADH.

Despite extensive investigation, the mechanism or mechanisms by which ethanol produces its effects on the central nervous system (CNS) remain far from resolved. Studies of the effect of ethanol on isolated axons have shown that ethanol is capable of producing a slight depolarization, decreasing the rate of rise of the action potential, and also decreasing the size of the action potential (168). Although these effects would be consistent with the depressant properties of ethanol, these alterations are only observed at concentrations which would be lethal to the intact animal. Similarly, ethanol has been shown to inhibit both the increase in intracellular sodium and the decrease in intracellular potassium caused by electrical stimulation of rat brain cortex slices (75) but again, at ethanol concentrations of 1.0 to 2.0%. Although drastic concentrations of ethanol are required to produce an observable change in these systems, smaller concentrations may produce alterations in these functions which, although unobservable, could still be important in the generation of ethanol's CNS effects *in* vivo.

In the last few years, interest has intensified in studies which suggest a common link between chronic ethanol and/or opiate use. For example, Ho *et al*. (68) have shown that in mice or rats chronically drinking ethanol, a single dose of morphine or methadone will decrease the amount of ethanol consumed. In addition, withdrawal of morphine from animals addicted to morphine increases their consumption of ethanol.

On the other hand, Blum et al. (21) have shown that morphine is able to decrease the severity of the ethanol withdrawal syndrome in mice while concurrent naloxone treatment during the course of chronic ethanol exposure decreased the resultant withdrawal syndrome when ethanol was discontinued (20). These authors speculate that these findings may be a reflection of the formation of tetrahydroisoquinoline alkaloids from condensation of aldehydes and catecholamines. It has been shown that acetaldehyde promotes the formation of tetrahydropapaveroline from condensation of dopamine and its first metabolite, dihydroxyphenylacetaldehyde in rat brain homogenates (43). This alkaloid has also been detected in vivo (161) after administration of ethanol and dopa, but not after a single dose of ethanol alone.

Another tetrahydroisoquinoline alkaloid, salsolinol, derived from direct condensation of acetaldehyde and dopamine, has been detected in the brain of rats treated with pyrogallol and ethanol but again not in the brains of rats administered ethanol alone (38). The findings that salsolinol depletes regional brain calcium (as do morphine and ethanol) (132) and that salsolinol has some opiate activity in the guinea pig ileum, (62) suggest that the link between these ethanol-derived alkaloids and opiates may be a viable one.

A major criticism of this link has been the failure to detect these alkaloids without prior pharmacological treatment. However, since these alkaloids are taken up by catecholamine neurons (64) they may be effectively concentrated and produce significant effects at very low whole brain concentrations. In addition, a recent report indicates that salsolinol is detectable in brains of mice exposed to ethanol vapour for

five days without prior treatment with either dopa or pyrogallol (61). As analytical methods improve it may be possible to evaluate the formation of these products even after a single dose of ethanol and thereby further investigate their role in the acute and chronic effects of ethanol especially as they relate to opiate interactions.

C) Ethanol and Other Drugs

The investigation of the effects of drug combinations has been of concern for many years. As one of the most frequently used drugs, the study of the effects of ethanol in combination with other drugs has naturally generated much interest, and several reviews of the field are available (50, 82, 125, 169). In addition to documentation of ethanol-drug interactions, investigators have started to ask how these interactions are produced. In this respect, the work of Rubin and Lieber and their coworkers has been of fundamental importance in emphasizing the ability of ethanol to inhibit the metabolism of many drugs both *in vivo* and *in vitro* (133).

Although ethanol decreases the *in vitro* metabolism of many drugs, it is not a uniform inhibitor of all drug metabolism. In general, ethanol is a more potent inhibitor of the metabolism of type II drugs such as aniline than of the metabolism of type I compounds such as pentobarbital (133). For example, an ethanol concentration of 12.5 mM reduced the p-hydroxylation of aniline to 50% of control, but an ethanol concentration of 660 mM was found necessary to produce the same degree of inhibition of the N-demethylation of ethylmorphine (35). Similarly, Liu *et al.* (93) found that the N-demethylation of benzphetamine, another type I drug,

was not altered by an ethanol concentration of 100 mM. These observations may be due to the fact that ethanol shows some similarities with type II compounds. Addition of ethanol to microsomes produces a spectral change similar to that produced by type II compounds (134), and also decreases the rate of NADPH-cytochrome P450 reductase (133). Ethanol has also been reported to decrease the binding of aniline but not hexobarbital (135). However, later work has demonstrated the ability of higher ethanol concentrations (100-200 mM) to decrease hexobarbital binding as well (32).

In addition to its effects on drug metabolism in vitro, an acute dose of ethanol is able to decrease the rate of elimination of a variety of drugs in vivo. Rubin et al. (133) showed that ethanol could decrease the rate of decline in whole blood-pentobarbital concentration in rats as well as in humans. The increased half-life of pentobarbital in rats was paralleled by an increase in the whole body half-life of pentobarbital which would indicate the alterations observed were not due solely to redistribution of drug. These workers also demonstrated an ethanol-mediated increase in the half-life of meprobamate in the blood of humans. In these studies they gave an initial ethanol dose of 1 g/kg followed by 24 g/subject every two hours thereafter and thus maintained a fairly high ethanol concentration for the duration of the drug half-life measurements. Carpenter et al. in a very complete analysis of ethanol-meprobamate interactions (24), found blood-meprobamate concentration in humans to be unaffected by a single dose of ethanol until at least 0.75 g/kg was administered. Similar studies in humans have failed to reveal an ethanol-mediated alteration in serum concentrations of oxazepam (99),

diazepam and thioridazine (90) and dilantin (138), perhaps since these experiments all employed an ethanol dose of 1.0 g/kg or less.

Studies in rats where higher doses of ethanol can be employed have shown significant alterations in drug concentration. For example, simultaneous intraperitoneal administration of glutethimide and 2.5 g/kg ethanol produced an initial decline in whole brain levels of glutethimide compared to control animals which was followed by a large increase in concentration 4 and 8 hours after the dose (67). It was also noted that ethanol treatment increased the brain/plasma ratio of glutethimide and caused an alteration in the within-brain distribution such that elevations in the pons-medulla concentration of glutethimide were greater than in the remainder of the brain.

Oral administration of ethanol 30 minutes prior to oral administration of diazepam produced initially lower levels of diazepam and metabolites in rat blood followed by concentrations greater than controls at later time periods (172). Ethanol also increased the whole brain concentration of diazepam and metabolites and increased the relative amounts of diazepam/metabolites in brain.

Combined intraperitoneal injection of ethanol and barbiturates has been shown to result in higher brain concentrations of pentobarbital (158) and phenobarbital (37) although the apparent half-life of pentobarbital in blood was not altered. Thus some of the increase in brain and blood barbiturate concentration may be due to increased absorption. Thomas *et al.* also noted that ethanol-treated rats excreted a smaller amount of pentobarbital metabolite (158), a finding similar to that of Coldwell *et al.* (36) who noted an increased concentration of phenobarbital and

decreased excretion of p-hydroxyphenobarbital in ethanol-treated rats. In none of the above studies was an alteration in the brain/blood distribution of barbiturate noted.

Mallov and Baesl (100) found that oral administration of ethanol two hours before intravenous administration of zoxazolamine or warfarin caused a significant increase in the plasma half-lives of these drugs in the rat. These authors attributed this increase to decreased microsomal metabolism of these drugs.

Chung and Brown (30, 31) have advanced the hypothesis that ethanol-mediated decreases in the rate of elimination of hexobarbital in vivo may be due not only to a direct effect of ethanol but also to an indirect inhibition of metabolism due to an ethanol-mediated increase in steroid release. They observed similar effects on elimination of hexobarbital in rats which were either administered acute stress by way of hindlimb ligature or ethanol. Further, inhibition of the metabolism of hexobarbital in 9000 X G supernatants after an in vivo dose of ethanol was only found in preparations from intact rats and not in preparations from adrenalectomized rats. These studies serve to illustrate that ethanol-mediated alterations in the brain concentration of various drugs may be produced by the sum of many different effects.

As might be expected, combination of ethanol with other CNS depressants generally results in an increased toxicity which is the result of addition of the individual effects of each drug. Accordingly, barbiturates (173) as well as glutethimide (104) and chloral hydrate (53) are more toxic in ethanol-treated animals than they are when administered to control animals. A similar increase in toxicity is observed with combi-

nations of ethanol and tranquillizers such as chlorpromazine, promazine and promethazine (48).

Combination of ethanol with drugs which have some CNS stimulant properties are less easy to predict. For example, even though ethanol decreases the metabolism and excretion of amphetamine, and may produce increased tissue levels of amphetamine, there is no alteration in the $LD_{50}$ of amphetamine (76). The results of administration of various mixtures of ethanol and chlorpheniramine show that low doses of ethanol actually antagonize the lethal effects of chlorpheniramine (145). These results may be a reflection of the ability of ethanol to counteract some of the stimulant effects of these drugs.

The combined toxicity of ethanol and opiates has been studied as well. Wagner and Wagner (166) found that the simultaneous subcutaneous administration of ethanol and methadone produced an increased toxicity which was approximately equal to the sum of the individual toxicities. The same conclusion was drawn by Eerola concerning the toxicity of ethanol and morphine (47). More recent investigations of the toxicity of a wide range of ethanol and morphine doses by McCoy et al. (97) revealed additive toxicity at high doses of ethanol but as observed with some stimulants, low doses of ethanol protected their mice from the lethal effects of morphine. The authors ascribe this protection to the depressant properties of ethanol counteracting some of the stimulant properties of morphine.

None of these investigators determined the concentration of drug in the CNS at the time of death and thus were not able to assess the impact, if any, of ethanol mediated alterations in drug disposition on the

observed results.

D) Other Drugs and Methadone

Although the effects of ethanol on the disposition of methadone have not been previously studied, the effect of other drugs on the metabolism and distribution of methadone has been examined. For example, chronic pretreatment of rats with phenobarbital has been shown to increase microsomal metabolism of methadone and to decrease the antinociceptive activity of methadone (3). Other workers have also shown that phenobarbital pretreatment increases the microsomal metabolism of methadone in vitro and suggest that this increased metabolism is responsible for the increased biliary excretion of methadone and methadone metabolites observed after chronic phenobarbital administration (129). In a similar vein, pentobarbital-pellet implantation has been shown to decrease the antinociceptive and toxic effects of methadone while increasing microsomal ethylmorphine N-demethylase activity in mice (69).

In addition to the barbiturates, chronic treatment with rifampin may have the ability to induce the metabolism of methadone. Kreek et al. (84) have shown that the combination of chronic rifampin treatment with methadone maintenance results in a decrease in plasma methadone concentration in humans when compared to periods when rifampin was not administered. However, the consistently lower plasma concentrations of methadone were not consistently accompanied by either shorter half-lives of methadone in plasma or consistent increases in excretion of methadone and metabolites, indicating that rifampin may alter methadone concentrations by other means in addition to microsomal enzyme induction.

Several agents have been shown capable of inhibiting methadone metabolism or increasing the half-life of methadone in vivo. Pretreatment of mice with SKF-525A has been shown to increase the concentration of methadone in plasma and several tissues when compared to animals receiving methadone alone (141). The *in vitro* metabolism of methadone is inhibited by diazepam (148), although pretreatment of mice with diazepam or oxazepam does not alter the brain or plasma concentration or half-life of methadone *in vivo* (142). Whether this difference is due to a species difference or to other *in vivo* effects of the benzodiazepines which mask alterations in the metabolism of methadone *in vivo* is not known. Liu and co-workers (92, 94) have found desipramine pretreatment capable of increasing both the antinociceptive effect and tissue concentration of methadone in rats. In addition, desipramine inhibits metabolism of methadone *in vitro* and inhibits the biliary and urinary excretion of methadone and its metabolites.

Thus, despite the existence of studies of the effects of ethanol on drug disposition and of the effects of various drugs on methadone disposition, there are no studies available on the effect of ethanol on the disposition of methadone. The need for such a study is indicated by the information available on the incidence of the combined use of these two agents.

E) Ethanol and Methadone

Although all methadone maintenance clinics collect random urine samples from their patients for the purpose of detecting drug use other than methadone, these clinics generally do not request an analysis for ethanol (163). There are many indications however, that alcohol use and alcoholism are significant problems in methadone maintenance patients. Analysis in our laboratory of a random group of 170 urine samples from methadone maintenance patients revealed that 18% had detectable levels of ethanol (greater than 0.02% w/v). Scott (140) reported that 25% of the methadone maintenance patients he studied were alcoholics by the standards of the National Council on Alcoholism, confirming a report (67) that in another program as many as 20% of the methadone maintenance patients were alcoholics.

Not only is the incidence of alcoholism high, but some authors suggest methadone maintenance may increase alcoholism. In a program where patients were admitted to methadone treatment only if not alcoholic, it was found that 34% were alcoholics after 4 years of methadone treatment (140). Another study found drinking to be one of the few significantly increased complaints during the course of methadone maintenance (118) which correlates well with the findings of another study which indicate that 70% of the problem drinkers in a methadone maintenance program became so after admission to treatment (98).

These reports suggest that the use of ethanol by heroin addicts increases during treatment in the methadone maintenance program. This impression is confirmed by the results of Schut (139) who found that of 100 patients, 68 drank "not at all" during daily use of narcotics, but

after 18 months of methadone treatment, only 38 still did not report drinking at all while 20 reported drinking "more" or "much more." A similar conclusion is drawn by Freedman (51). Both these authors note that alcohol use seems to increase during periods of methadone dose reduction (detoxification). There is not complete agreement about increased use of ethanol during methadone maintenance. One author claims a decrease in ethanol use (55). However, even in this study the overall incidence of heavy drinking was 10%.

The motivations for ethanol use in people using methadone are no doubt as diverse as the alcoholic drive in any population. However, the methadone user may have several additional reasons for ethanol use. In a series of interviews designed to determine the nature of the illicit or "street use" of methadone, Agar (1) found that 33% of the people who use another drug with methadone use wine to "boost the methadone high." The intentional concomitant use of ethanol to intensify the methadone effect was also noted by others (26,89). Other possible reasons for ethanol use in methadone maintenance patients could be: (1) to obtain a qualitatively different high since the patient is tolerant to the effect of narcotics, (2) decrease the side effects of methadone maintenance, or (3) as a device for access to a different social group.

Bihari (18) indicated that very often the amount of alcohol consumed by a methadone-maintenance alcoholic far exceeds that of a non-opiate alcoholic and that as a consequence, the development, and medical consequences of, alcoholism in these people is much more rapid and severe. The incidence of alcohol use and alcoholism in methadone maintenance patients has motivated several centers (27,89,126) to use

disulfiram in combination with methadone to treat their patients who are addicted to both ethanol and methadone.

An additional indication of the incidence of combined ethanol and methadone use comes from a report of drug findings in victims of non-drug-induced violent deaths in New York City and Detroit, Michigan (13). This study showed that of those homicide victims who were using morphine at the time of death, 20% were also using ethanol while of those who had been using methadone, 40% were also using ethanol. A final indication of the magnitude of the combined use of ethanol and methadone comes from reports of the findings in cases of methadone overdose. The frequency with which ethanol is found in published surveys of methadone overdoses varies. One study found that in sixteen cases of methadone overdose, thirteen had also ingested ethanol (26). This high incidence of ethanol in methadone deaths is not found in other studies (14, 101, 128) although ethanol is frequently associated with methadone deaths in these reports. A more reliable index of the involvement of ethanol in methadone deaths is obtained from the National Registry of Human Toxicology which is compiled from voluntarily submitted toxicology reports from the entire United States (111). The results in the Registry for 1973-1976 show that 20% of the reports of methadone-induced death also involved ethanol.

The degree of narcotic tolerance in the victims of methadone overdose is often hard to determine. Some are known to be tolerant, as they were enrolled in a methadone maintenance program. Others are thought to be non-tolerant by virtue of their recent confinement in a hospital or jail. In the remainder of the cases the victim may have been an

occasional or chronic user of illicit methadone.

One would expect the methadone maintenance patient to be less susceptible to methadone overdose, but there exists one report (130) that patients maintained for long periods of time on high doses of methadone have a much higher death rate when they are also addicted to ethanol.

Reporting on the results of analyses of methadone overdoses in New York City, Bastos (12) noted that the brain concentration of methadone in deaths due to ethanol and methadone tended to be lower than in cases of death attributed to methadone alone. However, the variability in the levels in both groups precluded a definite statement about the role of ethanol in these deaths. Much of the variability was probably due to the various degrees of narcotic tolerance in the cases examined, which cannot be controlled or even ascertained in human postmortem studies.

F) Present Study

This study was motivated by the information summarized above which indicates that there is a significant human exposure to combinations of ethanol and methadone. It is the purpose of this study to evaluate the nature and magnitude of some of the interactions of these two agents when administered in a controlled situation to laboratory animals. The interactions to be studied are summarized as follows:

1) Effect of ethanol on the pharmacological activity of methadone

Since methadone is both used and abused for its effects on the central nervous system, the most meaningful effect to monitor would be a CNS effect. As one of the most prominent and well studied CNS effects of methadone is its antinociceptive activity, this was the effect

investigated. The activity of many narcotic drugs in the tail-flick and hot-plate tests is well correlated with their clinical effectiveness in man (6) and for this reason they are two of the most widely used tests of antinociception. Therefore, the activity of methadone in the tail-flick and hot-plate tests was used as a measure of its pharmacological activity in this study.

2) Effect of ethanol on the magnitude and time course of brain methadone concentration

As summarized in the introduction, ethanol has the potential for producing significant alterations in the amount of systemically administered drug which reaches the CNS. In view of the possibility of ethanol mediated alterations in the absorption, distribution, metabolism or excretion of methadone, the concentration of methadone in the brain was determined simultaneously with antinociceptive activity. To reliably quantitate methadone in individual mouse brains, $^{3}$H-methadone was used in most of these experiments.

3) Toxicity of ethanol and methadone combinations

The antinociceptive and lethal properties of methadone are probably produced by effects on different systems and therefore, alterations in antinociception may not necessarily imply alterations in lethal effects. Thus, this study also investigated the effect of ethanol pretreatment on the toxicity of methadone as well as the effect of chronic methadone administration on the toxicity of ethanol.

# MATERIALS AND METHODS

## A) Materials

### 1) Drugs

The l-$^{3}$H-l-methadone HBr used in this study was purchased from New England Nuclear (NEN). The initial specific activity was 110 mCi/mmole. This was diluted with unlabled d,l-methadone HCl except where noted otherwise. The unlabled methadone was obtained from Mallinckrodt. Unlabled l-methadone was a gift from Eli Lilly as were authentic samples of the two major metabolites of methadone, EDDP and EMDP. Naloxone HCl was a gift from Endo Laboratories, and SKF-525A was a gift from Smith-Kline and French. Propoxyphene was purchased from Eli Lilly, morphine sulphate was purchased from Mallinckrodt, and meperidine HCl was purchased from Winthrop Laboratories. All ethanol solutions used in this study were prepared fresh from absolute ethyl alcohol U.S.P. purchased from U.S. Industrial Chemicals.

### 2) Chemicals

Omnifluor, Aquasol-2 and the $^{3}$H-toluene internal standard were purchased from New England Nuclear. Omnifluor (4 g/liter) was dissolved in Scintill AR toluene purchased from Mallinckrodt. All other chemicals used were of analytical reagent grade.

### 3) Animals

The mice used in these studies were male Swiss (ICR) mice weighing between 19 and 30 g. The rats employed were male Sprague Dawley weighing between 250 and 325 g. Animals were maintained on Purina Laboratory

Chow for rats and mice and tap water ad libitum in an animal room with a 12-hour light-dark cycle. Animals were purchased from Flow Research Animals, and were allowed to acclimatize for at least two days prior to experiment.

B) Methods

1) Dosing of Animals

Unless otherwise noted, all animals were deprived of food but not water, and placed in wire bottom cages at midnight the night before the experiment. All experiments were conducted between 4:00 and 8:00 pm the next day during which time the animals did not have access to either food or water. The start of the normal dark cycle was at 7:30 pm.

All drugs for subcutaneous injection were dissolved in 0.9% NaCl at a solvent volume of 10 ml/kg. All drug doses are expressed as the free base. Methadone administered by the oral route was dissolved in deionized water. Ethanol was administered to rats as a 24% (w/v) solution in deionized water. Low-power microscope examination revealed that this concentration of ethanol produced reddening and pinpoint hemorrhaging of the gastric mucosa. For this reason all subsequent ethanol doses were administered as a 15% (w/v) solution. At this concentration there was no observable damage to the mucosa. Control animals received an equal volume of deionized water.

Since the gross behavior of the animals was still affected by a 2.5 g/kg dose of ethanol 30 minutes after administration, but the animals appeared normal at 60 minutes, most of the studies of the antinociceptive

effects of ethanol and methadone combinations were done at 60 minutes or more after the ethanol dose.

Radiolabeled methadone was diluted with unlabeled methadone to produce a specific activity such that animals injected subcutaneously received 5 to 10 μCi/kg (most frequently at a specific activity of 5 μCi/mg) and animals dosed orally received 40 μCi/kg. Subcutaneous injections were made under the skin between the shoulder blades.

Drug administrations were spaced to allow sufficient time to sacrifice and obtain samples from each mouse immediately after testing and still maintain the same time interval between drug injections and testing for all mice. Unless otherwise noted, mice were dosed and tested only once. Animals were killed by cervical dislocation. Blood was withdrawn from the heart with a 25-gauge needle and allowed to clot in a capped test tube. Clotted blood was centrifuged and the serum was refrigerated. In cases where whole blood was analyzed, it was collected over potassium oxalate and sodium fluoride. The brain was rapidly removed, blotted free of surface blood, wrapped in aluminum foil, and placed in a beaker in an ice bath. Liver and lung samples were removed and placed on dry ice. All tissue samples were stored frozen until analysis.

2) Analysis of Injection Solutions

All $^{3}H$-methadone solutions were stored at $4^{o}C$. Any methadone solution more than six months old was checked for concentration and radiochemical purity before use. Concentration was checked by dilution with 0.5 HCl and measurement of UV absorption at 259 nm. Radiochemical purity was determined by extraction of an alkaline aliquot with 50 μl of

ethylene dichloride/isopropanol (80/20, v/v) and thin layer chromatographic analysis (TLC) in the solvent system described below. The TLC plate was scribed prior to application of the sample to provide channels approximately 1 cm wide. After development, the zone corresponding to methadone was scraped into a vial and the remainder of the channel was scraped into a second vial. Isopropanol (0.5 ml) and Omnifluor in toluene (10 ml) were added and the amount of radioactivity was determined. In addition, aliquots of the injection solution were used to prepare standards for the analysis of samples. Aliquots of the extracted aqueous layers were counted to monitor exchange of tritium. At no time in the course of the study was any degradation of methadone or exchange of label observed in the injection solutions.

3) Measurement of Antinociception

The primary technique for the measurement of antinociception was the modified (11) tail-flick test of D'Amour and Smith (42). In this test the animal's tail was placed on a notch above a photocell so as to block the photocell. A photographic lamp mounted above the photocell was focused on the tail approximately 3 cm from the end. Activation of the lamp also started a timer placed in series. When the animal perceived the heat from the lamp and moved his tail, light from the lamp hit the photocell and stopped the timer. A rheostat was used to adjust the intensity of the lamp so that undosed animals flicked their tails between two and four seconds after activation of the lamp.

A control or baseline latency was obtained for each mouse before dosing. At the appropriate time after drug administration, the latency

of the tail-flick was again determined. If animals allowed their tail to remain under the lamp for more than ten seconds, the heat produced marked tissue damage. For this reason a cutoff of ten seconds was employed. Antinociceptive effects were calculated as percent of the maximum possible effect (%MPE) according to this formula:

$$\%MPE = \frac{\text{Test Latency} - \text{Baseline Latency}}{10 - \text{Baseline Latency}} \times 100.$$

In addition, when animals responded with a test latency less than ten seconds, their tails were replaced over the photocell with the lamp off to confirm that they would not move their tails within ten seconds in the absence of a painful stimulus. This was done to decrease the contribution of possible random movement to the calculated antinociceptive response. In fact, it was found that unless the animals were held in a very awkward position they very rarely displayed any random tail movements. In the cases where this was found, the animal was replaced in the cage for one to two minutes, replaced on the tail-flick apparatus with the light off for ten seconds, and then tested.

The second measure of antinociception was the hot-plate test of Eddy and Leimbach (46). The mouse was placed in a clear plastic cylinder approximately 4" x 12" which was on a brass plate maintained at 57°C by a recirculating water bath. The time required for the mouse to react to the heat by licking its front paws or lifting one of his rear paws was measured with a stopwatch. A control reading was taken before drug administration. The maximum time allowed was twenty seconds. The antinociceptive effect (%MPE) was calculated according to the following formula:

$$\%MPE = \frac{\text{Test Latency} - \text{Baseline Latency}}{20 - \text{Baseline Latency}} \times 100.$$

Antagonism of the antinociceptive effect of methadone, or ethanol and methadone, by naloxone was determined by administration of various doses of naloxone to animals pretreated with ethanol and methadone in doses sufficient to produce approximately 80% MPE. Percent antagonism was calculated as follows:

$$\%\text{antagonism} = 1 - \frac{\%\text{MPE with naloxone}}{\%\text{MPE without naloxone}} \times 100.$$

4) Determination of Methadone Concentrations

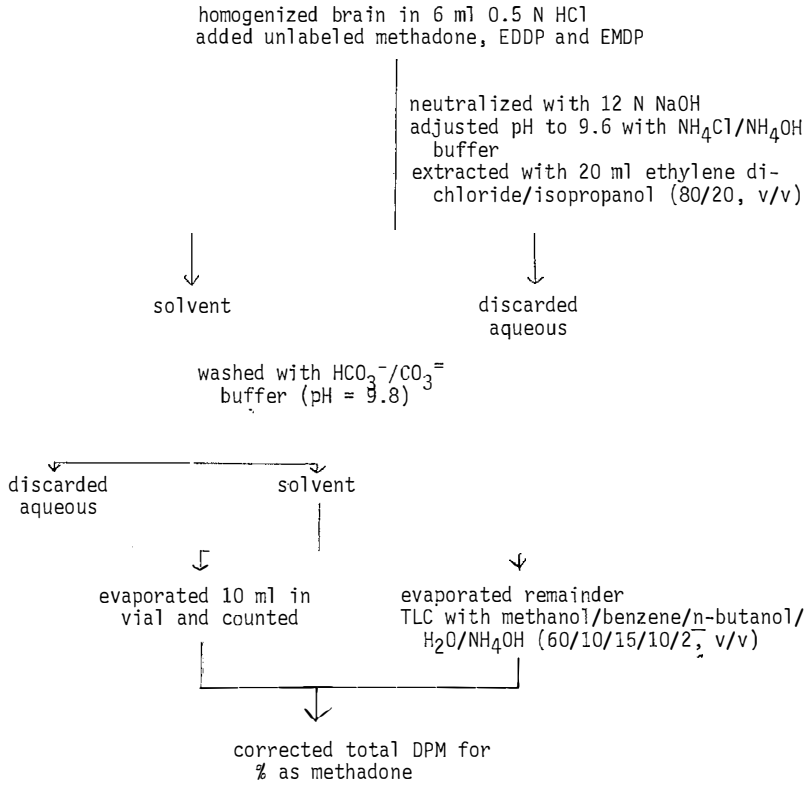
a) Analysis of $^{3}H$-methadone in brain

Whole brains were homogenized in 2 ml 0.5 N HCl with a glass and teflon tissue grinder (A.H. Thomas). The homogenizer was washed with 2 x 2 ml 0.5 N HCl. The combined homogenate and washings were spiked with 500 µg of unlabeled methadone and 25 µg of both EDDP and EMDP. The extraction scheme, adapted from the method of Misra et al. (108), is presented in figure 2. Each brain was homogenized and extracted separately. The pH 9.6 $NH_4Cl/NH_4OH$ buffer was prepared by adjusting a saturated solution of $NH_4Cl$ to pH 9.6 with 15 M $NH_4OH$.

Ten ml of the solvent extract from each brain or brain standard was evaporated in an individual vial. The residue in the vials was dissolved in 0.5 ml of isopropanol and 10 ml of Omnifluor in toluene was added. The remaining solvent extract from all brains which came from the same treatment group of mice was pooled in an evaporation cup and evaporated at room temperature under a slow air stream. The residue was dissolved in 3.0 ml of 0.3 N HCl and transferred to a tapered centrifuge tube. The evaporation cup was then washed with an additional 1 ml of 0.3 N HCl. The combined acid washings were adjusted to pH 9.6 with

FIGURE 2

PROCEDURE FOR THE DETERMINATION OF RADIOLABELED METHADONE IN BRAIN



the $NH_4Cl/NH_4OH$ buffer and then vortexed with 100 µl of ethylene dichloride/isopropanol (80/20,v/v). The organic solution was applied to a Silica Gel G (250 microns) plate which had been previously scribed into 1 cm channels, and developed in MeOH/benzene/n-butanol/$H_2O$/$NH_4OH$ (60/10/15/10/2, v/v) adapted from Beckett et al. (16). The zone corresponding to methadone was scraped into one vial and the remainder of the channel was placed in another vial. Isopropanol and Omnifluor were added as described above. Radioactivity in all samples was determined in a Beckman LS 300 scintillation counter and corrected for quenching by the external standard method. The accuracy of the quench curve was checked by also using $^3$H-toluene as an internal standard in several samples from each batch. The results of both methods were in good agreement.

The apparent methadone in each brain was calculated by comparison of the total DPM in the 10 ml solvent aliquot from samples to the number of DPM in the 10 ml of solvent from blank mouse brain standards that had been spiked with known amounts of $^3$H-methadone from the injection solution. It was found that methadone was extracted from human-brain homogenates to the same extent as from mouse-brain homogenates and thus only one standard per batch was prepared from mouse brain, the remaining standards were prepared from human-brain homogenates. At least two standards were run with each ten samples. Brain methadone was calculated by the following formula in which the apparent brain methadone was corrected for the percent of total $^3$H which migrated with methadone on TLC:

$$\text{Apparent methadone (\% as methadone)} \cdot \frac{\text{Brain Weight}}{\text{}} = \text{ng methadone/gram of brain.}$$

For example: "300 ng" (0.85)/0.45 g = 567 ng/g.

The coefficient of variation for thirty-one standards extracted within a seven-week period was 7%. The recovery of added methadone from both human and mouse-brain standards was consistently greater than 95%.

TLC zones were visualized with iodoplatinate spray (33). Some increased sensitivity was observed when the spot corresponding to EMDP was sprayed with Dragendorff spray (33) instead of iodoplatinate. The $R_f$ of EDDP, methadone and EMDP were 0.21, 0.50 and 0.75 respectively.

The recovery of $^3$H-methadone spotted on the TLC plate was only approximately 75% for both extracted samples and standards as well as an ethanol solution of methadone applied directly to the plate. The losses were assumed to be due to incomplete removal of the drug from the silica gel particles. To see if the extraction procedure or the loss of $^3$H during TLC would affect the determination of brain methadone an experiment was carried out with pooled urine from animals that had received $^3$H-methadone. The pooled urine was split into two portions. One was analyzed by gas liquid chromatography (GLC), the other was added to two blank mouse-brain homogenates and carried through the above procedures. The GLC analysis revealed a 1/0.29/0.07 ratio (based on triplicate injections) between methadone/EDDP/EMDP while the average of DPM in the corresponding TLC zones was 1/0.32/0.03. Thus, even though there is some apparent loss of $^3$H on the TLC plate, the relative amount of methadone to its metabolites is not altered and therefore the correction of apparent brain methadone to actual brain methadone should not be altered.

b) GLC analysis of methadone in brain

In addition to the analysis of brain methadone by the use of

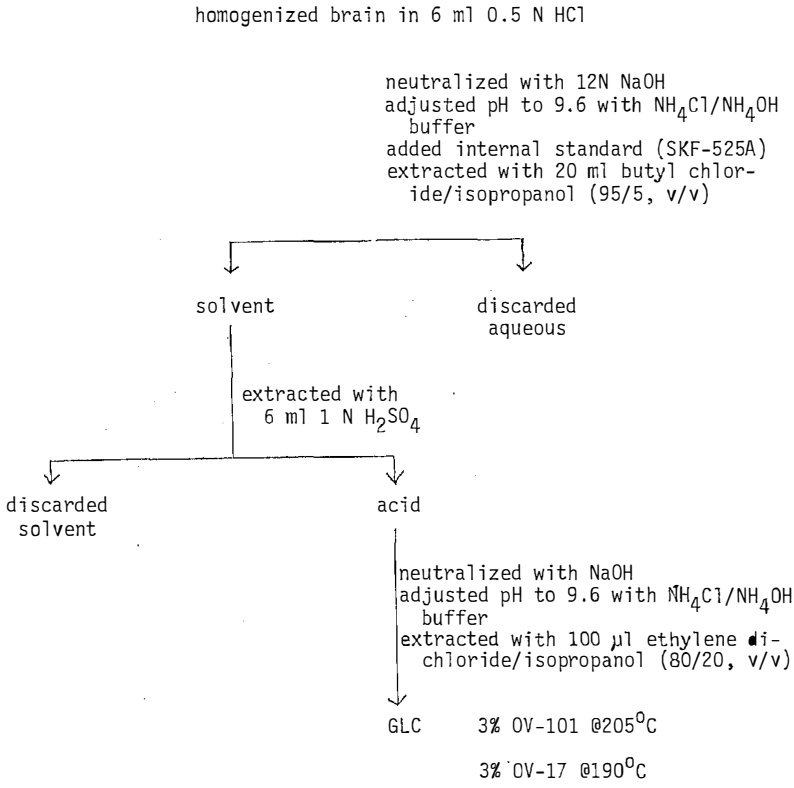
$^{3}$H-methadone, it was necessary to develop a GLC procedure for brain methadone determinations for two reasons. First, during the course of these experiments, it was found that the specific activity of methadone in the brain was altered when $^{3}$H-methadone was administered by the oral route. A more complete discussion of this problem is presented in appendix I. In addition, experiments were conducted in mice maintained on unlabeled methadone where use of radiolabeled drug would not be possible. The GLC method employed was a modification of a procedure previously published from this laboratory (2).

Although not affected by alterations in specific activity, the GLC method was less sensitive than the assay based upon the $^{3}$H-label, and therefore brains had to be pooled in groups of at least two. The brains were homogenized in the same manner as in the $^{3}$H-label assay but were not spiked with additional methadone or metabolites. Fifty micrograms of the internal standard SKF 525A (beta-diethylaminoethyldiphenylpropylacetate HCl) was added to the brain homogenate prior to pH adjustment. This compound has been used by others (73) as an internal standard for methadone analysis due to its structural similarity to methadone. The extraction scheme is presented in figure 3.

The final extract was analyzed on a Bendix 3300 gas chromatograph with flame ionization detectors. Each sample was injected on both a 3% OV-101 and a 3% OV-17 column to minimize the effect of any contaminants which would coelute with either methadone or the internal standard on one column or the other. The peak heights and areas as well retention times and methadone/SKF-525A ratios were determined by a Hewlett Packard 2100 computer-based data reduction system. The amount of methadone in

FIGURE 3

PROCEDURE FOR THE DETERMINATION OF METHADONE IN BRAIN
BY GAS LIQUID CHROMATOGRAPHY



each sample was calculated by comparison of peak height ratios with coextracted brain standards spiked with known aliquots of the injection solution. At least one standard was run for every four samples. The results from both columns were in good agreement and were averaged together for each sample.

The overall recovery of methadone was 85%. The major methadone metabolites were poorly back extracted by $H_2SO_4$ but any metabolites in the final extract did not interfere with the methadone peak under the chromatographic conditions employed. Based on 5 standards, the within-run coefficient of variation was 6% at a level of 1 μg. The minimum amount that could be reliably quantitated was 100 ng.

In contrast to the extraction of methadone, the recovery of the internal standard was not the same from mouse and human-brain homogenates. The internal standard recovery from human-brain homogenates was almost exactly twice the recovery from mouse brains and thus comparison of mouse-brain samples with human-brain standards would give an apparent brain concentration of methadone approximately twice as high as it should be (see appendix I). For this reason, in addition to standards prepared from human-brain homogenates, at least one standard prepared in blank mouse brains was run with each batch of samples.

In addition, two analyses of pooled brain samples analyzed by this GLC method were also analyzed by a gas chromatograph/mass spectrometer (GC/MS). This confirmation was performed on an electron impact, magnetic sector mass spectrometer (Du Pont 490B) set to monitor m/e 72 which is the base peak of methadone and a minor peak in SKF 525A. The GC/MS results were in very good agreement with GLC results, the average

difference between the two being 7%. The GC/MS was also used to detect any residual brain methadone at twenty-four hours after the last maintenance dose in methadone-tolerant mice. In the single ion detection mode the instrument was capable of detecting 5-10 ng of methadone.

c) Determination of methadone in serum, liver and lung

Serum was diluted with water. Approximately 0.4 g of liver or lung were homogenized in 0.5 N HCl. These samples were processed by the procedure described for brain. Liver and lung samples were removed from the freezer and homogenized immediately to minimize any alterations of the relative amounts of methadone and metabolites due to metabolism in vitro.

d) Determination of liver methadone and metabolites

The determination of the relative amounts of methadone and its metabolites in liver was accomplished by preparing and extracting samples as described above. However, instead of scraping all the non-methadone area of the TLC channel into one vial, zones corresponding to methadone, EDDP and EMDP were scraped into separate vials. The remainder of the channel was then placed in a separate vial. In addition, a known aliquot of the extracted aqueous phase was also counted. The amount of $^{3}H$ present as methadone, EDDP or EMDP was calculated by multiplying the total extractable $^{3}H$ by the percentage of total radioactivity on the TLC plate which migrated with each metabolite. The total radioactivity in the organic solvent and extracted aqueous layers were summed. The ratio of total radioactivity to the radioactivity present as methadone or each metabolite or the radioactivity remaining in the aqueous phase was then determined.

Analysis of the final organic solvent extract from liver was also performed using iTLC sheets (type SA) purchased from Gelman. These fiberglass sheets impregnated with silica gel were developed in either benzene/ethyl acetate/methanol/$NH_4OH$ (80/20/1.2/0.1, v/v) or t-amyl alcohol/n-butyl ether/water (14/17/1, v/v) according to the procedure of Misra et al. (109). After development, the strips were cut into 10-mm sections, placed in vials with 1 ml of isopropanol and then counted as described above.

e) Methadone and metabolites in bile and urine

Bile and urine samples from each treatment group were pooled, diluted with the $NH_4Cl/NH_4OH$ buffer, spiked with 30 µg of methadone, EDDP and EMDP, and vortexed with 200 µl of EDC/isopropanol (80/20). The organic solution was applied to a TLC plate and developed as described above. The samples were not hydrolyzed but simple solvent extraction still removed 80% of the radioactivity from the urine layer 60 minutes after methadone administration.

5) Determination of Ethanol Concentrations

a) Sample preparation

Serum was analyzed without further preparation. Brain samples were removed from the freezer one at a time, weighed, and immediately homogenized in 2 ml of cold deionized water. The homogenate was transferred to a 5 ml volumetric flask. The homogenizer was washed with another 2 ml of cold water which was combined with the homogenate. The combined homogenate and wash was diluted to a volume of 5 ml with cold water. The flask was sealed and the contents thoroughly mixed. Brain

samples were analyzed within 12 hours of the experiment as longer periods of storage tended to allow some loss of ethanol even when frozen.

Samples of stomach contents were obtained by ligation of the esophagus and duodenum followed by removal of the stomach from the mouse. The stomach was opened and the contents washed into a 25-ml volumetric flask with approximately 20 ml of cold deionized water. The level was brought to the mark with cold water, the flask stoppered, and the contents thoroughly mixed.

b) Analysis

Ethanol samples were analyzed by an automated head space analysis using a Perkin Elmer gas chromatograph (Multifract F-40). A known aliquot of the sample (500 ul) was combined in a 20 ml vial with 4.5 ml of an aqueous solution of n-propanol which serves as an internal standard. The vial was sealed and incubated at 58°C for at least 20 minutes. An aliquot of the head space was automatically injected on a Carbopack C column at 110°C. The ratio of the ethanol peak height to the n-propanol peak height was determined by computer and the concentration of ethanol in the sample calculated by comparison to aqueous standards. The total amount of ethanol in the brain sample thus calculated was divided by the initial brain weight to yield the concentration of ethanol in the brain. For example, if 381 mg of brain was homogenized in 5 ml of water and this solution was found to contain a total of 0.602 mg of ethanol, then the brain contained 1.58 mg/g or 158 mg/100g. The concentration of ethanol in the aqueous standards was determined by a modified Cavett titration (114). The validity of using aqueous standards

in the analysis of brain samples was verified by adding known amounts of ethanol to blank mouse brains and preparing and analyzing them as described. The coefficient of variation for the ethanol analysis is less than 2% based on analysis of thirty aliquots of a blood sample containing 0.10% ethanol (w/v).

6) Locomotor Activity

The locomotor activity cages used consisted of a clear plastic shoebox cage with a photocell at one end and a white light focused on it from the other end. The cage was covered by a wire screen. There were six such cages housed in a metal cabinet with a fan to circulate air and serve as a constant "white noise" background. The photocells were connected to an analogue counter assembly (Lehigh Valley Electronics) so that the number of times the mouse interrupted the light beam could be quantitated. Two mice were placed in each cage and the doors to the cabinet were closed. After 10 minutes, the counter was activated and the number of counts during the next 30 minutes was recorded.

7) Statistics

All $ED_{50}$'s, $ID_{50}$'s and $EC_{50}$'s and their 95% confidence intervals were calculated and tested for significant potency and slope ratios by the method of Litchfield and Wilcoxon (91). All other data with exception of binomial data such as %MPE was tested by the two-tailed *t* test (150). Binomial data (%MPE) were evaluated for significant differences by the Mann-Whitney U test (39). The only exception was in the evaluation of the effect of ethanol alone on the tail-flick and hot-plate.

In this case the absolute latencies before and after ethanol were tested for significant ($P < .05$) differences by the *t* test. This is valid since absolute latency is a continuous, random variable as long as no animal reaches the ten second cutoff.

# RESULTS

## A) Antinociceptive Activity of Ethanol

The antinociceptive effect of ethanol 60 minutes after administration to male Sprague Dawley rats is presented in table 1. Increasing oral doses of ethanol produced increasing whole brain ethanol levels, however significant effects on tail-flick latency were not observed until a dose of 4.5 g/kg was employed.

The antinociceptive effect of various doses of ethanol in ICR mice is presented in table 2. The serum and whole brain concentrations of ethanol follow the kinetics typical of a substance whose metabolism is described by zero order kinetics. After a dose of 2.5 g/kg brain-ethanol levels fell at a rate of 48 mg/100 g/hour. No significant alteration of tail-flick latency was observed from 30 to 150 minutes following an ethanol dose of 2.5 g/kg.

Following an oral dose of 2.5 g/kg of ethanol mice appeared to have decreased locomotor and exploratory activity as well as reduced skeletal muscle coordination. These effects were maximal 30 minutes after the dose. At 60 minutes after administration, ethanol-pretreated mice exhibited the same behavior and muscle coordination as control mice.

Although the end point in the hot-plate test requires a more coordinated motor response than the tail-flick, mice pretreated with 2.5 g/kg of ethanol showed no increase in hot-plate latency at 60 minutes. Since an oral dose of 2.5 g/kg of ethanol did not elicit any activity in either the hot-plate or tail-flick tests it was the dose of ethanol used in most of the interaction studies.

TABLE 1

TAIL-FLICK ACTIVITY OF ETHANOL IN RATS

| Dose (g/kg, p.o.) | %MPE Tail Flick | Whole Blood Ethanol mg/100 ml ± SEM | Brain Ethanol mg/100 g ± SEM |
|---|---|---|---|
| 1.5 | 4.8 | 151 ± 15 | ---- |
| 2.5 | 3.0 | 169 ± 39 | 161 ± 38 |
| 3.0 | 5.0 | 266 ± 27 | ---- |
| 4.5 | 10.8* | 273 ± 45 | ---- |

Animals were dosed with ethanol (24% w/v) p.o. and tested 60 minutes later. There were four rats/dose.

* significantly different from zero at $P < .05$

TABLE 2

TAIL-FLICK ACTIVITY OF ETHANOL IN MICE

| Dose g/kg | Time After Dose (Minutes) | Serum Ethanol mg/100 ml ± SEM | Brain Ethanol mg/100 g ± SEM | %MPE Tail-Flick |
|---|---|---|---|---|
| 2.5 | 30 | - | 215 ± 9 | 5.2 |
| | 45 | 252 ± 56 | 205 ± 15 | -1.0 |
| | 60 | 222 ± 40 | 193 ± 7[a] | 2.0[b] |
| | 90 | 198 ± 21 | 167 ± 4 | 1.0 |
| | 120 | 167 ± 29 | 148 ± 8 | 0.1 |
| | 150 | 140 ± 19 | 123 ± 10 | 3.0 |
| 4.0 | 60 | 413 ± 67 | 319 ± 21 | 13 |
| | 90 | 341 ± 41 | 261 ± 14 | 10 |
| | 120 | 311 ± 33 | - | 5 |
| | 150 | 285 ± 36 | 223 ± 16 | 0.5 |
| 6.0 | 60 | 675 ± 51 | 500 ± 45 | 85* |

Ethanol was administered p.o. as a 15% w/v solution. Mice were sacrificed immediately after testing. There were at least six mice/group.

[a] Combination of this dose of ethanol with simultaneous administration of methadone 4 mg/kg s.c. resulted in a brain-ethanol concentration of 197 ± 18 mg/100g.

[b] These mice also displayed an antinociceptive effect of -5% MPE in the hot-plate test.

* Significantly different from zero at $P < .05$.

As shown in table 2, ethanol at a dose of 4.0 g/kg did produce some elevation of tail-flick latency. In addition, at this dose the effects of ethanol on muscle coordination and locomotor activity were more pronounced and long lasting. When tested on the hot-plate, the mice appeared to perceive the heat but were unable to coordinate the normal response. At a dose of 6.0 g/kg mice were extremely sedated and exhibited a very marked elevation of tail-flick latency as well as a total lack of spontaneous movement.

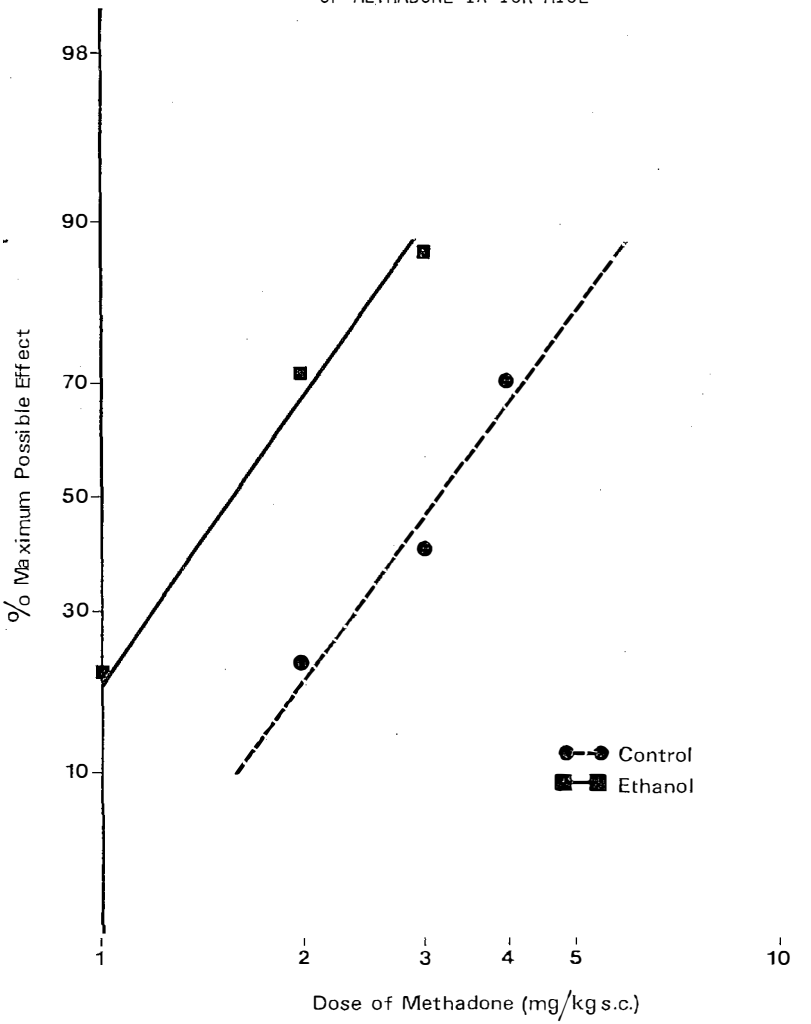
B) Effect of Ethanol on Methadone Antinociception

As shown in figure 4, a 2.5 g/kg dose of ethanol, which is not active in the hot-plate test alone, when combined with methadone produces a significant difference in the antinociceptive effect of methadone. The test was done 60 minutes after ethanol at which time the mice had recovered from the effect of ethanol on skeletal muscle coordination and displayed no change of latency due to ethanol alone. The $ED_{50}$ of methadone is decreased from 3.2 mg/kg in control mice to 1.6 mg/kg when combined with ethanol. There is no significant difference in the slopes of the dose response curves but the $ED_{50}$'s are significantly different at the 95% confidence level by the method of Litchfield and Wilcoxon (91).

The combination of ethanol and methadone was also more active than methadone alone in the tail-flick test as shown in figure 5. There was no alteration of the tail-flick response by an ethanol dose of 2.5 g/kg. Yet, when combined with methadone this dose of ethanol decreased the $ED_{50}$ of methadone in the tail-flick test from 2.0 mg/kg to 0.8 mg/kg. Furthermore, when pretreated with 4.0 g/kg ethanol, mice displayed a methadone $ED_{50}$ of only 0.2 mg/kg. The slopes of the dose response curves

FIGURE 4.

THE EFFECT OF ETHANOL ON THE HOT-PLATE ACTIVITY OF METHADONE IN ICR MICE

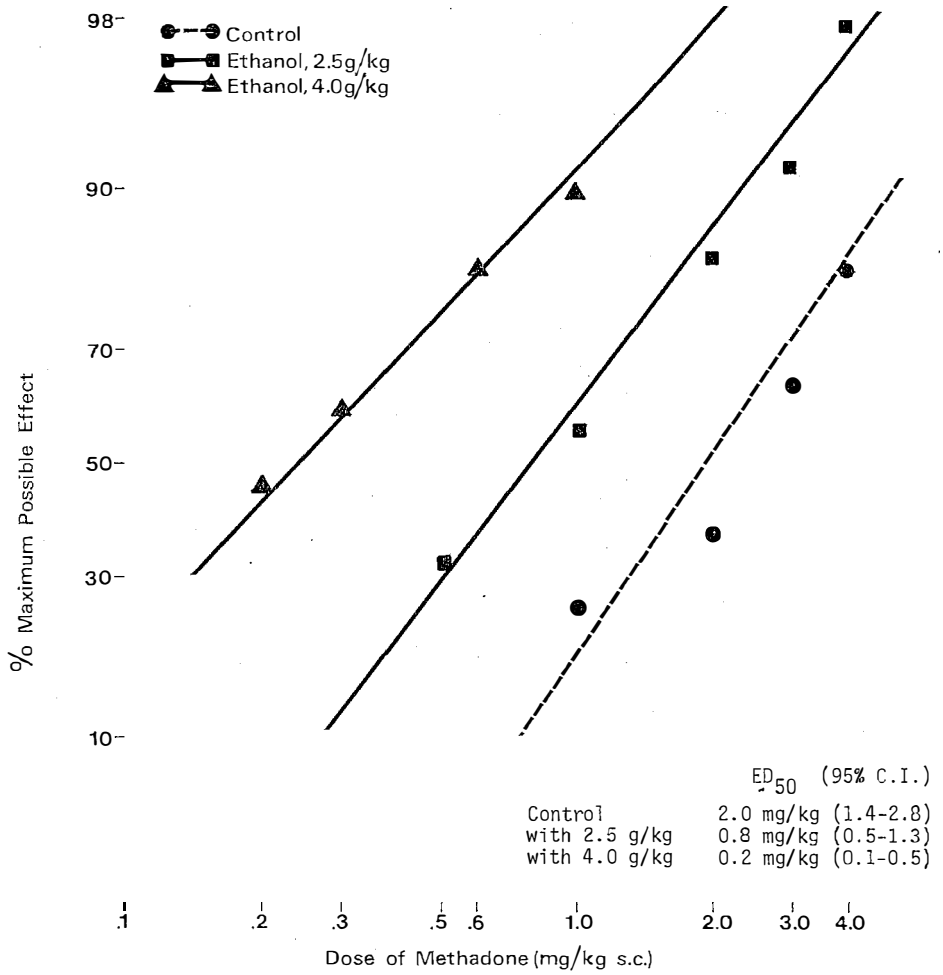



Control $ED_{50}$ = 3.2 (2.3-4.5), with Ethanol $ED_{50}$ = 1.6 (1.0-2.5)

Water or Ethanol (2.5 g/kg, p.o.) was administered 45 minutes prior to methadone. Mice were tested 15 minutes post methadone. N = 6/group.

FIGURE 5

THE EFFECT OF ETHANOL PRETREATMENT ON THE TAIL-FLICK ACTIVITY OF METHADONE




Ethanol (2.5 or 4.0 g/kg, p.o.) or water was administered 45 minutes prior to methadone and mice were tested 15 minutes later. N = at least six mice/group.

were similar but the alterations of methadone potency were significantly different at the 95% confidence level by the method of Litchfield and Wilcoxon (91). Thus, these data indicate that ethanol produces a dose-related increase in methadone antinociception as measured in both the hot-plate and tail-flick tests.

As shown in figure 6, a similar result is found in Sprague Dawley rats. Pretreatment with a dose of ethanol not active in the tail-flick test (2.5 g/kg, see table I) produced a significant potentiation of methadone antinociception as evidenced by a decline in the $ED_{50}$ of methadone from 4.3 mg/kg to 2.2 mg/kg, with no change in slope.

C) Effect of Ethanol on Methadone Distribution

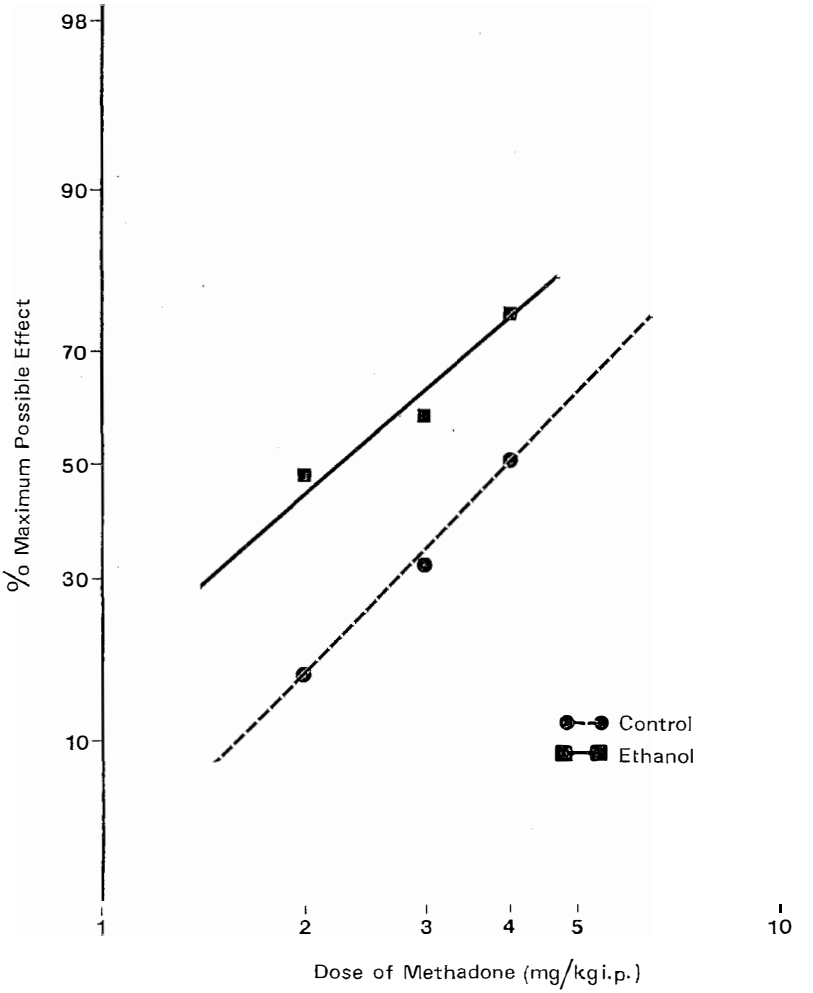
1) Methadone Concentrations in Brain and Serum

To examine the possibility that the increased antinociception was due to an increased concentration of methadone in the brain, the concentration of $^{3}H$-methadone in the brain and serum of the mice used to construct the preceding dose-response curves was examined. The results of these determinations are presented in table 3. It can be seen that at each dose examined, the mice which received ethanol had a lower concentration of methadone in the brain but a greater antinociceptive response compared to the water-pretreated controls. With the exception of the combination of water and 4.0 mg/kg methadone, the brain to serum methadone concentration ratio was approximately 1.0 and was not altered by ethanol pretreatment.

As can be seen in the last column of this table the brain-methadone concentration in ethanol-pretreated mice was decreased by approximately 20% compared to the water controls. However, ethanol potentiated

FIGURE 6

THE EFFECT OF ETHANOL ON THE TAIL-FLICK ACTIVITY OF METHADONE IN SPRAGUE DAWLEY RATS




Control $ED_{50}$ = 4.3 (3.1-6.0), with Ethanol $ED_{50}$ = 2.2 (1.5-3.0)

Ethanol (2.5 g/kg, p.o.) or water was administered 45 minutes prior to methadone. Animals were tested 15 minutes post methadone. N = 6 animals/group.

TABLE 3

EFFECT OF ETHANOL ON BRAIN AND SERUM METHADONE CONCENTRATION

| Dose of Methadone mg/kg (s.c.) | Ethanol Pretreatment | | | Water Pretreatment | | | Ratio of Brain Levels @ |
|---|---|---|---|---|---|---|---|
| | % MPE # | Methadone Level ng/g ± SEM | | % MPE # | Methadone Level ng/g ± SEM | | |
| | | Brain | Serum | | Brain | Serum | |
| 0.5 | 32 | 57±8 | 63±12 | -- | 70±13 | 83±17 | 0.81 |
| 1.0 | 62 | 139±8 | 156±15 | 25 | 159±8 | 151±12 | 0.87 |
| 2.0 | 81 | 309±27 | 284±27 | 38 | 334±18 | 357±30 | 0.93 |
| 3.0 | 92 | 388±36 * | 493±31 | 64 | 508±31 | 510±83 | 0.76 |
| 4.0 | 100 | 642±55 * | 651±103 | 80 | 857±65 | 550±89 | 0.75 |

Ethanol (2.5 g/kg, p.o.) or water was administered 45 minutes prior to Methadone. Mice were sacrificed 15 minutes later. Methadone levels were determined by liquid scintillation. N = at least 6 mice/group. * significantly different from control at $P < .05$

@ Ratio of brain methadone concentration in ethanol pretreated mice/water pretreated mice
# analgesic effect in the tail flick test.

methadone to such an extent that even in the face of this slight decrease in brain methadone, the dose-response curve for methadone was still shifted to the left (figure 5).

The effect of 4.0 g/kg ethanol on brain methadone was also examined. A dose of 0.5 or 1.0 mg/kg methadone combined with 4.0 g/kg ethanol produced brain concentrations of methadone of 62 ng/g $\pm$ 13 and 140 ng/g $\pm$ 25 respectively. Thus, even though a 4.0 g/kg dose of ethanol produced higher brain and serum levels of ethanol than 2.5 g/kg, the effect on methadone concentration in the brain is the same.

2) Changes in Absorption and Distribution to Peripheral Tissues

In order to investigate the reasons behind the decreased brain level of methadone when combined with ethanol, studies of the effects of ethanol on the absorption of methadone from the subcutaneous injection site and the distribution of methadone to other tissues were conducted. The injection site and underlying musculature were excised and washed twice with 0.5 N HCl. An aliquot of the acid was mixed with Aquasol-2 and raidoactivity was measured. It was found that 30 minutes after a 4.0 mg/kg dose, 90 and 92% of the dose had been absorbed from the injection sites in ethanol and water-pretreated animals respectively.

Another possible reason for decreased concentrations of methadone in brain was an ethanol-induced increase in distribution of methadone to other tissues. Levels of $^{3}$H-methadone in brain, serum, liver and lung were determined at various times after a 4.0 mg/kg dose of methadone following either water or ethanol (2.5 g/kg) pretreatment. The results of these determinations are presented in table 4. As observed in the dose-response experiment (table 3), ethanol produced a significant decrease in brain and serum methadone compared to control animals. However, this

TABLE 4

THE EFFECT OF ETHANOL ON THE DISTRIBUTION OF METHADONE AND UNCONJUGATED METABOLITES

| Tissue | Time After Methadone Administration(1) (Min.) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | 30 | | 90 | | 150 | | 210 | |
| | ETOH | Control | ETOH | Control | ETOH | Control | ETOH | Control |
| Brain | | | | | | | | |
| Total(2) | 8.19 ± .37** | 11.2 ± .07 | 3.20 ± .12** | 6.49 ± .30 | .94 ± .15* | 1.31 ± .07 | 0.58 ± .09 | 0.63 ± .03 |
| Methadone(3) | 6.96 ± .31** | 9.7 ± 0.6 | 2.66 ± .10** | 5.41 ± .25 | .71 ± .11* | 1.05 ± .06 | 0.44 ± .07 | 0.49 ± .02 |
| Serum | | | | | | | | |
| Total | 8.81 ± .20** | 13.6 ± 0.9 | 3.29 ± .25** | 6.41 ± .31 | 0.92 ± .10 | 1.16 ± .10 | 0.59 ± .08 | 0.48 ± .04 |
| Methadone | 7.05 ± .25** | 10.5 ± 0.7 | 2.51 ± .09** | 5.00 ± .35 | 0.70 ± .12 | 0.83 ± .07 | 0.43 ± .06 | 0.35 ± .03 |
| Liver | | | | | | | | |
| Total | 265 ± 19 | 201 ± 23 | 149 ± 13 | 125 ± 15 | 62.1 ± 3.1 | 56.0 ± 15 | 36.5 ± 2.5 | 42.1 ± 4.1 |
| Methadone | 92.6 ± 12 | 80.3 ± 11 | 59.6 ± 3.7 | 49.8 ± 3.1 | 25.1 ± 1.3 | 23.7 ± 1.8 | 15.0 ± 1.8 | 13.1 ± 2.8 |
| Lung | | | | | | | | |
| Total | 348 ± 21 | 338 ± 25 | 105 ± 7.4 | 99.1 ± 6.5 | 52.0 ± 3.9 | 45.3 ± 5.3 | 44.1 ± 5.6* | 30.9 ± 2.8 |
| Methadone | 310 ± 19 | 301 ± 22 | 88.9 ± 6.3 | 80.3 ± 5.3 | 41.1 ± 3.1 | 36.2 ± 4.2 | 35.3 ± 4.5* | 24.7 ± 2.2 |

(1). Ethanol or water was administered p.o. 30 minutes before Methadone, 4 mg/kg s.c.
(2). Values are extractable DPMX10$^{-3}$/g of tissue wet weight ±SEM, N = 6 animals/group.
(3). Unmetabolized Methadone was calculated by correcting total extractable $^{3}H$ for the % which migrated with Methadone on TLC as described in Methods.

* different from control at P<.05 ;** different from control at P<.01

was not accompanied by uniform significant increases in either liver or lung concentrations of methadone although at each time point investigated the ethanol-pretreated mice had slightly greater methadone concentrations in these tissues. This finding agrees with the data on subcutaneous absorption since, if ethanol decreased brain-methadone concentrations only by decreased absorption, then liver and lung concentrations would be expected to be lowered also. When liver and lung concentrations of methadone were examined in relation to the serum concentration of methadone perfusing them, (table 5) it was seen that ethanol pretreatment produced a significant increase in hepatic and pulmonary uptake of methadone.

In this experiment, as in all others where the brain/serum distribution of methadone was examined, both brain and serum were found to contain approximately equal concentrations of methadone. The only exception was in the dose-response experiment (table 3) where 4 mg/kg methadone in control mice gave a brain/serum ratio of 1.6. However, the experiment presented in table 4 shows that with this dose at 30 minutes post methadone, the brain/serum ratio is 0.92, indicating that there is probably no real dose-dependent alteration in methadone distribution between brain and serum.

The relative amounts of total $^{3}H$ to $^{3}H$-methadone in brain and serum shown in table 4 are representative of the ratios determined in other experiments. There was no alteration in the relative amounts of $^{3}H$-methadone to metabolites in brains from mice treated with 0, 2.5, or 4.0 g/kg ethanol. These ratios were used to calculate methadone concentration from total extractable $^{3}H$ values as described in methods. As expected, the liver contained the greatest relative amount of methadone

TABLE 5

EFFECT OF ETHANOL ON LIVER/SERUM AND LUNG/SERUM METHADONE RATIOS

| | Time After Methadone Administration (min) | | | |
|---|---|---|---|---|
| | 30 | 90 | 150 | 210 |
| Liver/Serum | | | | |
| Ethanol | 13.1 ± 1.5* | 23.7 ± 4.8* | 35.9 ± 4.1 | 34.9 ± 3.2 |
| Control | 7.6 ± 1.0 | 9.96 ± 1.2 | 28.6 ± 3.2 | 37.4 ± 3.6 |
| Lung/Serum | | | | |
| Ethanol | 43.8 ± 4.8* | 35.4 ± 4.3** | 58.7 ± 4.1* | 82.1 ± 4.9 |
| Control | 28.7 ± 3.2 | 16.1 ± 2.0 | 43.6 ± 5.1 | 70.6 ± 10.3 |

Values are the ratios of methadone concentrations from Table 4.

*Significantly different from control at $P < .05$

**Significantly different from control at $P < .01$

metabolites of the tissues examined.

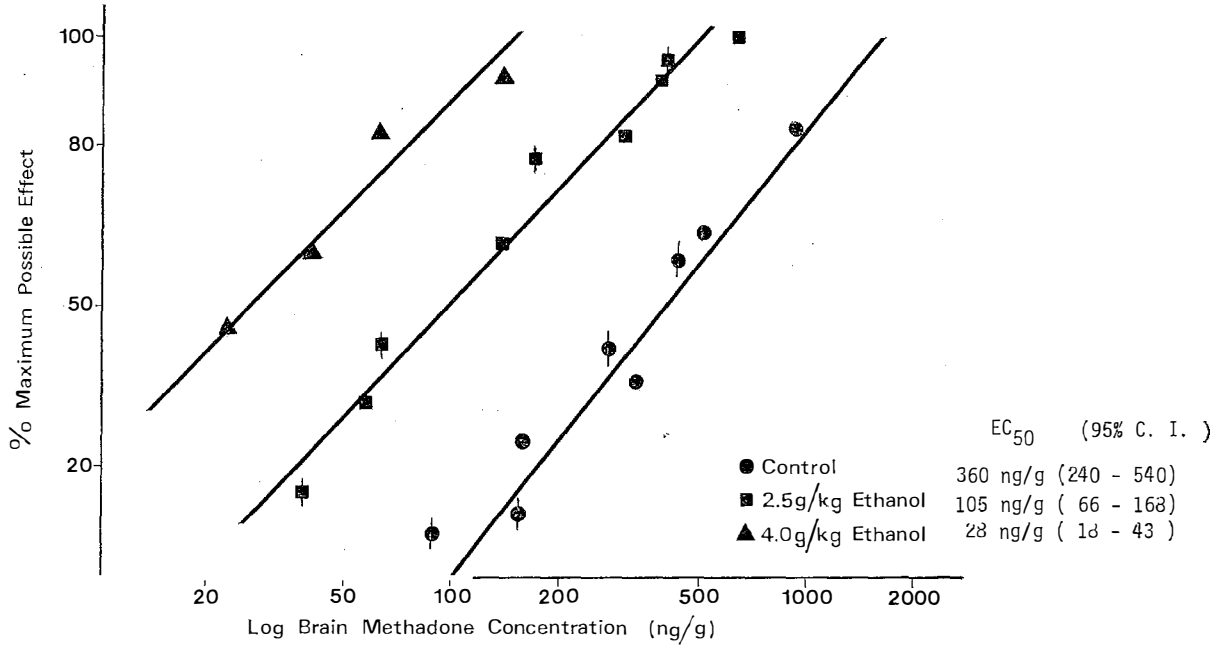
D) Correlation Between Brain Concentration of Methadone and Antinociceptive Effect.

In view of the effect of ethanol on the amount of methadone in the brain, a brain concentration-response curve may be a more valid measure of ethanol potentiation of methadone antinociception than the dose-response curve. The least square lines relating brain methadone to effect in animals administered methadone 15 minutes and 60 minutes prior to testing were colinear and therefore, the data from both dose-response curves was used to construct the concentration-response curves presented in figure 7. The concentration-response curve for 4.0 g/kg ethanol was obtained 15 minutes post methadone.

In all three cases, pretreatment with water, 2.5 or 4.0 g/kg ethanol, there is a very high correlation between whole brain methadone and antinociceptive effect. The correlation coefficients are 0.94, 0.92 and 0.89 respectively. At any given brain concentration of methadone the antinociceptive effect is greater in ethanol pretreated mice. The Litchfield and Wilcoxon $EC_{50}$'s for control, 2.5 g/kg and 4.0 g/kg ethanol treated mice are presented in figure 7. Ethanol pretreatment with a 2.5 g/kg dose produced an approximate threefold parallel shift in the concentration-response curve while pretreatment with 4.0 g/kg produced an additional threefold shift to the left. These data show that the increased antinociceptive activity of methadone in ethanol-pretreated animals was not due to an increased methadone concentration in brain.

FIGURE 7.

CORRELATION BETWEEN BRAIN CONCENTRATION OF METHADONE AND TAIL-FLICK ACTIVITY




Ethanol ( 2.5 or 4.0 g/kg, p.o.) or water was administered 60 minutes prior to test. Methadone was administered s.c. 15 or 60 minutes prior to test. Brain methadone levels were determined by liquid scintillation. N = at least six mice/group.

● ■ = points from the dose response curve 60 minutes post methadone
all other points are from the dose response curve at 15 minutes post methadone

E) Effect of Ethanol on the Time Course of Methadone Antinociception

1) Time course following subcutaneous administration

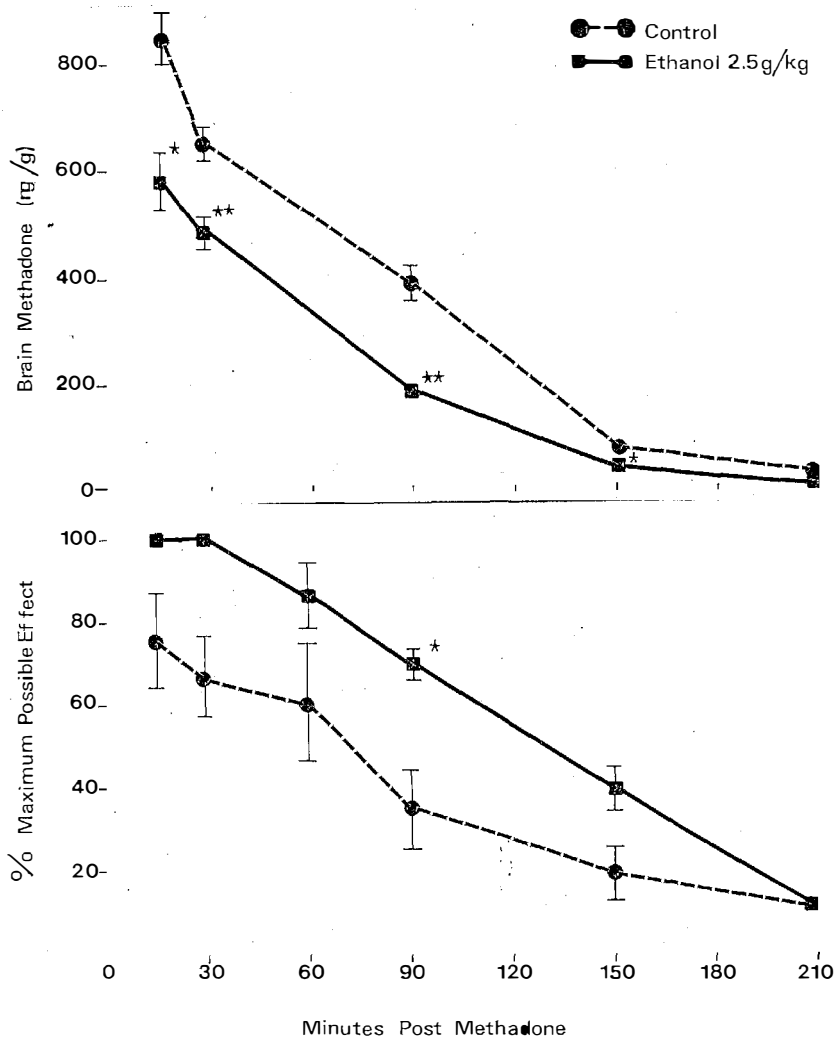
Mice were adminstered either water or ethanol and injected with methadone 30 minutes later. The effects of ethanol on methadone antinociception and brain level are presented in figure 8. As was found in the dose-response studies, ethanol-pretreated mice had lower brain concentrations of methadone but exhibited a greater antinociceptive response at every time point studied. Animals tested and sacrificed at 210 minutes were also tested for antinociception at 60 minutes post methadone. Brain concentrations of methadone were lowered to approximately the same extent as found in the dose-response experiments. In addition, the relationship between brain level and antinociceptive effect both with and without ethanol was similar to that presented in figure 7.

Since the antinociceptive response to methadone alone at 4 mg/kg was greater than 60% MPE for 60 minutes and the maximum possible effect is 100% MPE the potentiation of methadone by ethanol is better examined at lower doses of methadone. In figure 9 mice were orally dosed with ethanol and then immediately administered methadone (2 mg/kg) subcutaneously. In this case the effect of methadone and ethanol was greater than methadone alone throughout the time course investigated. The degree of potentiation seemed greatest at 60 minutes following methadone. Since the brain level and effect of both ethanol and methadone were changing during this experiment, potentiation was studied under conditions where the effect of ethanol was constant.

Results of the administration of ethanol at a uniform 60 minutes

FIGURE 8.

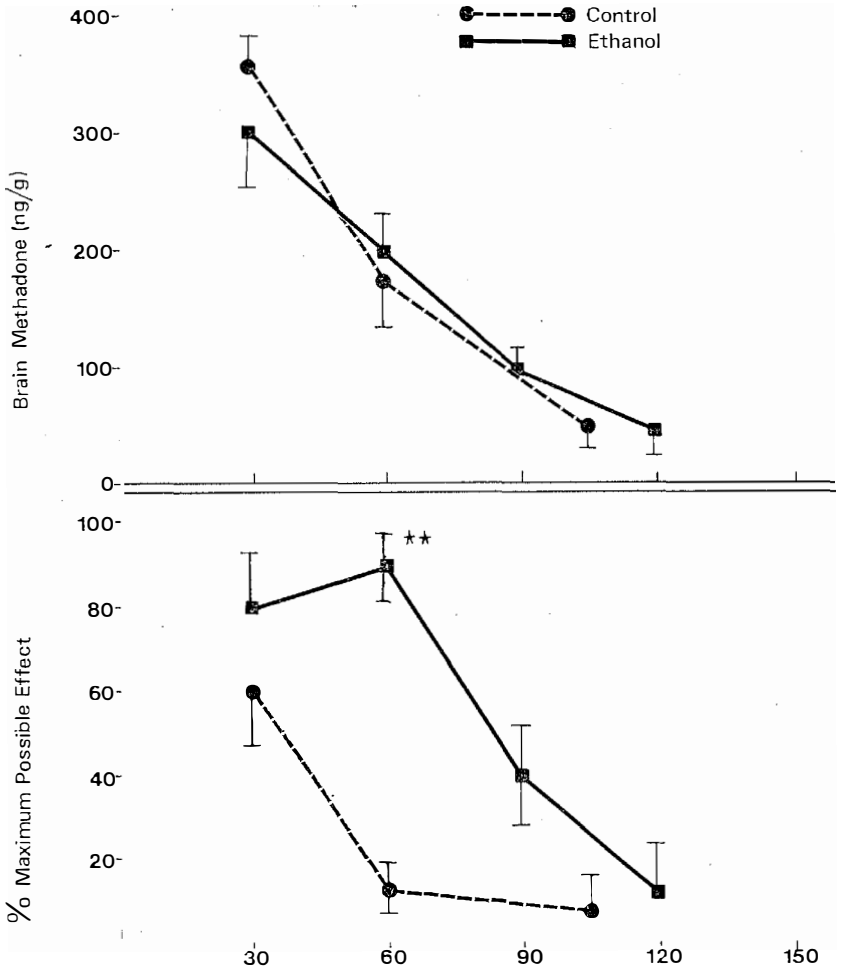
EFFECT OF ETHANOL ON THE TIME COURSE OF TAIL-FLICK ACTIVITY AND THE CONCENTRATION OF METHADONE IN BRAIN




Water or Ethanol(2.5 g/kg, p.o.) was administered 30 minutes prior to methadone. Brain levels determined by liquid scintillation. Each point represents the mean ± SEM of six mice.
* significantly different from control at $P < .05$
** significantly different from control at $P < .01$

FIGURE 9.

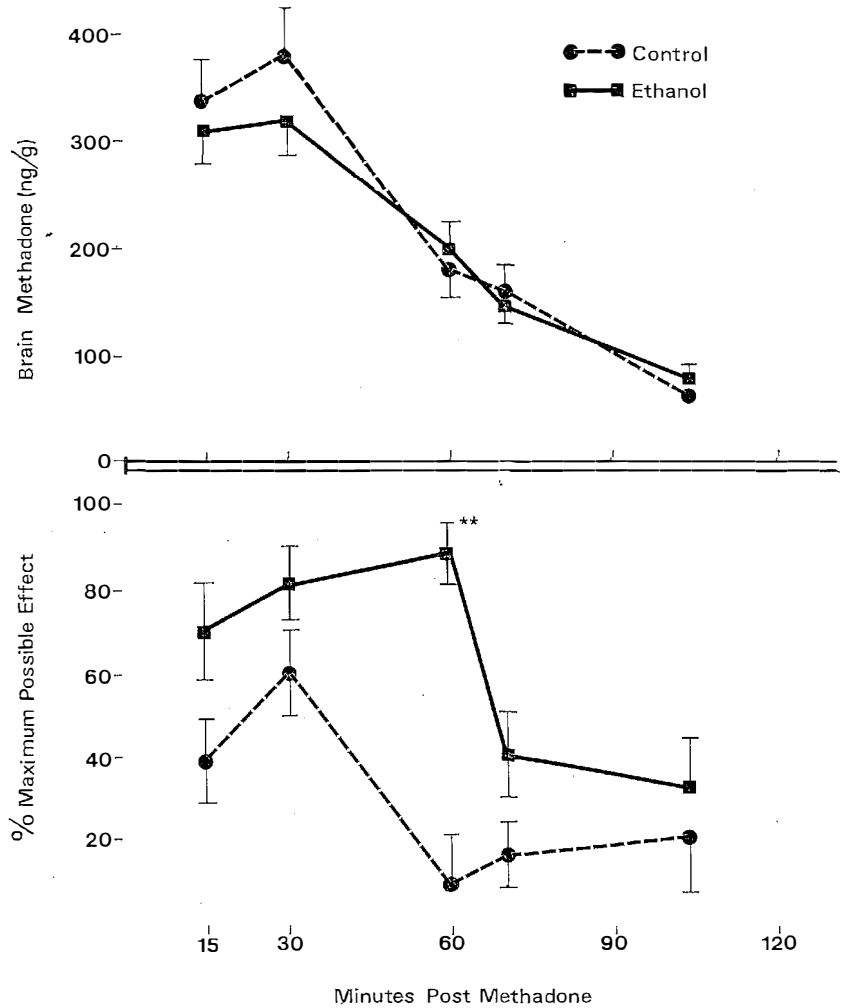
EFFECT OF ETHANOL ON SIMULTANEOUSLY ADMINISTERED METHADONE




Water or ethanol (2.5 g/kg, p.o.) administered at same time as methadone (2 mg/kg, s.c.). Brain levels of methadone were determined by liquid scintillation. Each point represents the mean ± SEM of at least six mice. ** significantly different from control at $P < .01$

FIGURE 10

THE TIME COURSE OF METHADONE TAIL FLICK ACTIVITY WHEN ETHANOL IS ADMINISTERED 60 MINUTES PRIOR TO TESTING




Methadone (2 mg/kg) was administered s.c. and animals were tested at various times thereafter. In all cases either ethanol (2.5 g/kg, p.o.) or water was administered 60 minutes prior to testing. Brain methadone was determined by liquid scintillation. There were at least 6 mice/group.

** significantly different from control at $P < .01$

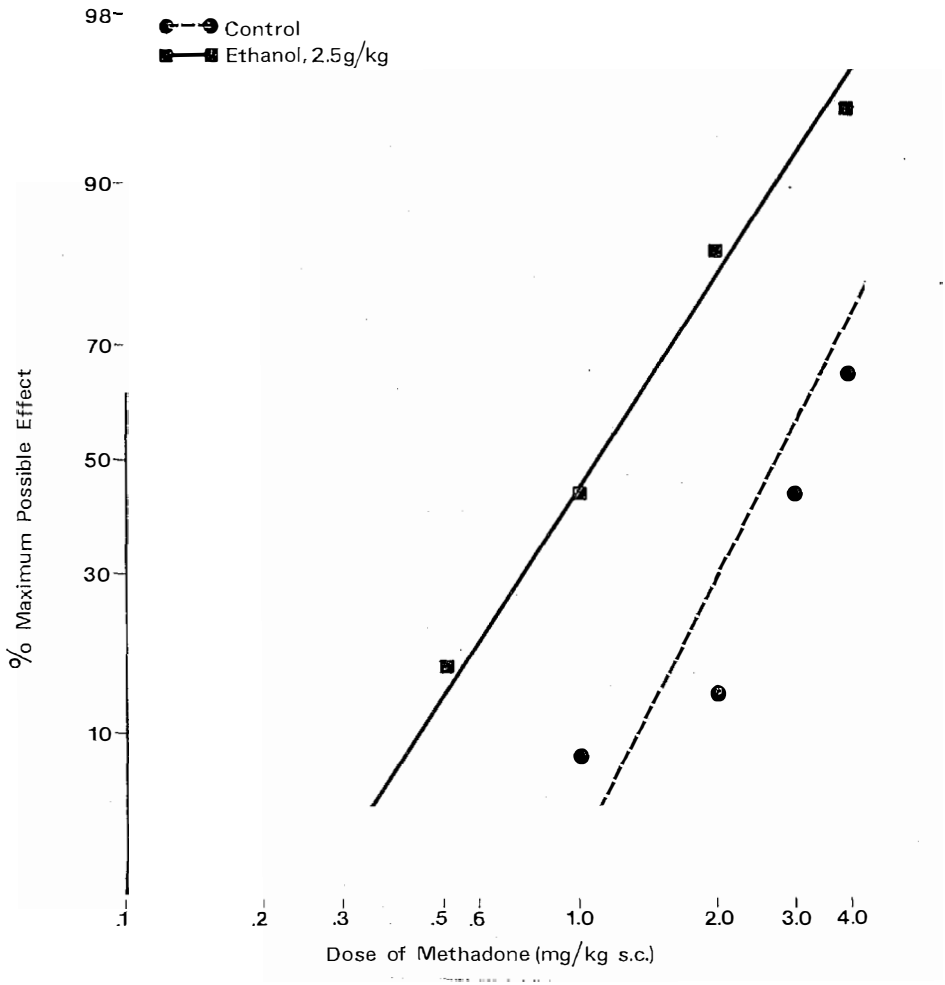
prior to testing and at various times after subcutaneous dose of methadone (2 mg/kg) are presented in figure 10. In this protocol the points at 15 minutes post methadone represent mice dosed with ethanol or water 45 minutes prior to methadone and tested 15 minutes after methadone. Similarly, the points at 105 minutes post methadone represent mice administered methadone 45 minutes prior to ethanol or water and tested 60 minutes later.

The data on brain concentrations of methadone indicate that, as before, when ethanol was administered prior to methadone, the ethanol-pretreated mice achieved lower methadone levels than their water-pretreated controls. However, when methadone was administered concomitantly or prior to ethanol, the two groups achieved similar brain concentrations of methadone. When the antinociceptive response is corrected for alterations in brain methadone level between the two groups, ethanol produced an approximate twofold increase at all time periods with the exception of 60 minutes post methadone. As in figure 9, the relative potentiation seems to be greatest when ethanol and methadone are administered simultaneously and tested at 60 minutes post methadone. Since the antinociceptive effect is on a binomial scale, the ratio of effect with ethanol/effect without ethanol is not the best way to gauge relative potency. In order to determine if the potentiation at 60 minutes post ethanol and methadone is greater than at other time intervals, the dose response curves in figure 11 were determined.

In this experiment, ethanol (2.5 g/kg) was administered orally and various subcutaneous doses of methadone were administered at the same time. In this protocol the potency ratio between methadone alone,

FIGURE 11.

THE EFFECT OF ETHANOL ON THE TAIL-FLICK ACTIVITY OF SIMULTANEOUSLY ADMINISTERED METHADONE




Ethanol (2.5 g/kg, p.o.) or water was coadministered with methadone and mice were tested 60 minutes later. N = six/group.

Control $ED_{50}$ = 2.8 (1.8-4.5) with ethanol $ED_{50}$ = 1.0 (0.6-1.6)

and ethanol and methadone was 2.8 whereas the potency ratio 15 minutes post methadone with a 45 minute ethanol pretreatment was 2.5 (figure 5). The differences in potency ratio as well as differences in ethanol or water pretreated $ED_{50}$'s between these two experiments were not significant. The concentrations of methadone in the brain and serum from these mice are presented in table 6. When ethanol and methadone were administered simultaneously, the brain concentration of methadone in ethanol-treated and control mice were generally similar.

In order to investigate whether the ability of ethanol to potentiate methadone declines more rapidly than brain-ethanol levels, three groups of mice were treated with 2.5 g/kg ethanol 90 minutes before subcutaneous administration of methadone (2 mg/kg) and tested at 60, 90 and 120 minutes post methadone (i.e. 150, 180 and 210 minutes post ethanol) when the estimated brain ethanol levels would be 140, 116, and 92 mg/100 g respectively. The data presented in the first two horizontal lines of table 7 show that ethanol is able to potentiate methadone as well at 150-210 minutes post ethanol as it is at 60-120 minutes. The decreased antinociceptive effect between the above treatments is probably solely due to the lesser ethanol levels during the test interval when ethanol is administered 90 minutes prior to methadone, rather than simultaneously with methadone.

2) Time course following oral administration

Since in most human use of ethanol and methadone the route of administration is oral rather than parenteral, interactions were also studied following this route of administration. Figure 12 presents the results of oral administration of ethanol or water 30 minutes prior to

TABLE 6

EFFECT OF ETHANOL ON BRAIN AND SERUM METHADONE CONCENTRATIONS

| Dose of Methadone mg/kg (s.c.) | Ethanol Pretreatment | | | Water Pretreatment | | | Ratio of Brain Levels[a] |
|---|---|---|---|---|---|---|---|
| | % MPE[b] | Methadone Level ng/g ± SEM Brain | Serum | % MPE[b] | Methadone Level ng/g ± SEM Brain | Serum | |
| 0.5 | 12 | 37±10 | 43±8 | -- | -- | -- | -- |
| 1.0 | 43 | 63±12 | 109±14 | 8 | 89±10 | 111±12 | 0.71 |
| 2.0 | 80 | 168±18 | 170±23 | 12 | 154±16 | 137±12 | 1.09 |
| 3.0 | -- | -- | -- | 45 | 281±36 | 302±53 | -- |
| 4.0 | 95 | 374±28 | 397±39 | 65 | 387±43 | 387±50 | 0.97 |

Ethanol (2.5 g/kg, p.o.) or water was coadministered with methadone. Methadone levels were determined by liquid scintillation. N= six mice/group.

[a]Ratio of brain concentration of methadone in ethanol pretreated mice/water pretreated mice

[b]Antinociceptive effect in the tail-flick test.

TABLE 7

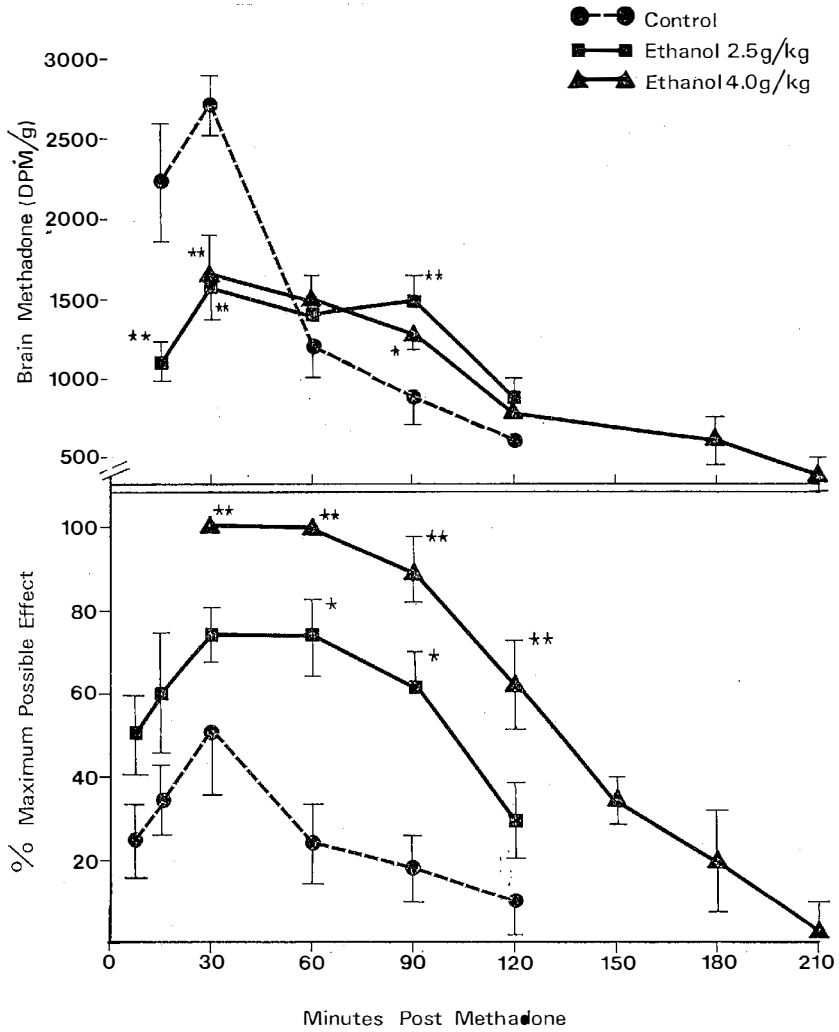
COMPARISON OF SIMULTANEOUSLY ADMINISTERED ETHANOL WITH 90 MINUTE ETHANOL PRETREATMENT

| | % MPE in the Tail-Flick Test | | |
|---|---|---|---|
| Time Post Methadone (min) | 60 | 90 | 120 |
| Ethanol and Methadone Coadministered (figure 9) | 90 | 40 | 10 |
| Ethanol 90 minutes before Methadone | 67 | 36 | 10 |
| Water and Methadone (figure 9) | 10 | -- | -- |

There were at least six mice per group.

FIGURE 12

EFFECT OF ETHANOL ADMINISTERED BEFORE METHADONE




Ethanol (2.5 or 4.0 g/kg, p.o.) or water was administered 30 minutes prior to methadone (8 mg/kg. p.o.). Each point represents the mean ± SEM of at least six mice per group.

* significantly different from control at $P < .05$
** significantly different from control at $P < .01$

oral administration of methadone. Since the mice had been deprived of food for 16 hours prior to drug administration, the absorption of methadone was rapid in the control animals. A significant antinociceptive effect was noted as early as 7 minutes after methadone was administered. Mice tested at 7 minutes were retested and sacrificed at 120 minutes. Mice tested at 150 minutes were retested and sacrificed at 210 minutes. In control animals peak concentrations of methadone in brain were achieved at 30 minutes post methadone. Pretreatment with ethanol seemed to delay the initial absorption of methadone but at times after 60 minutes the ethanol-pretreated animals had a greater methadone concentration in brain than the control animals. As was noted following subcutaneous administration, the effect of ethanol on the concentration of methadone in brain was not dose related. The effect of 4.0 g/kg ethanol on whole brain concentrations of methadone was the same as 2.5 g/kg ethanol. However, the increase in antinociceptive response is dependent upon the dose of ethanol administered. There was once again a strong correlation between brain methadone and antinociceptive response in all three groups. Since there was significant alteration in the specific activity of $^{3}$H-methadone following oral administration, all brain levels, although still corrected for the amount of methadone metabolites present, are reported as DPM/g (see appendix 1).

Although some of the alterations in the concentration of methadone in the brain following this route of administration are presumably due to the same effect noted following subcutaneous administration, there seems to be an additional effect of ethanol upon the absorption of orally administered methadone. To investigate this, the amount of methadone remaining in the stomach at various times after administration was deter-

mined. As can be seen in table 8, there is a rapid loss of methadone from the stomach in control animals. The loss of methadone is delayed following a dose of 2.5 g/kg ethanol. The same delay is observed following pretreatment with 4.0 g/kg ethanol. This loss of methadone from the stomach reflects the sum of absorption from the stomach as well as emptying of the stomach contents into the small intestine. The effect of ethanol outlasts the presence of ethanol in the stomach since by 60 minutes following both 2.5 and 4.0 g/kg doses, approximately 95% of the ethanol had left the stomach. Thus, in addition to the effect of ethanol on methadone once it is absorbed, ethanol also causes an initial delay in methadone absorption following oral administration.

In order to examine the potentiation of orally administered methadone without these significant alterations in the concentration of methadone in brain methadone was administered orally 30 minutes prior to ethanol. At this time approximately 90% of the methadone dose had left the stomach and thus alterations of the absorption of methadone would be expected to be less. Figure 13 shows that with this dosing protocol there were no significant alterations in the amount of methadone in brain.

F) The Effect of Food Deprivation on Methadone Antinociception.

To investigate the possibility that the ethanol potentiation of methadone antinociception is mediated by its ability to alter blood glucose levels, experiments in which the nutritional status of the mice was changed were undertaken. The results of these manipulations are presented in table 9.

Administration of ethanol to free-feeding mice would be expected to slightly elevate blood glucose concentrations (7) however, administration of isocaloric glucose (7) instead of ethanol did not alter methadone

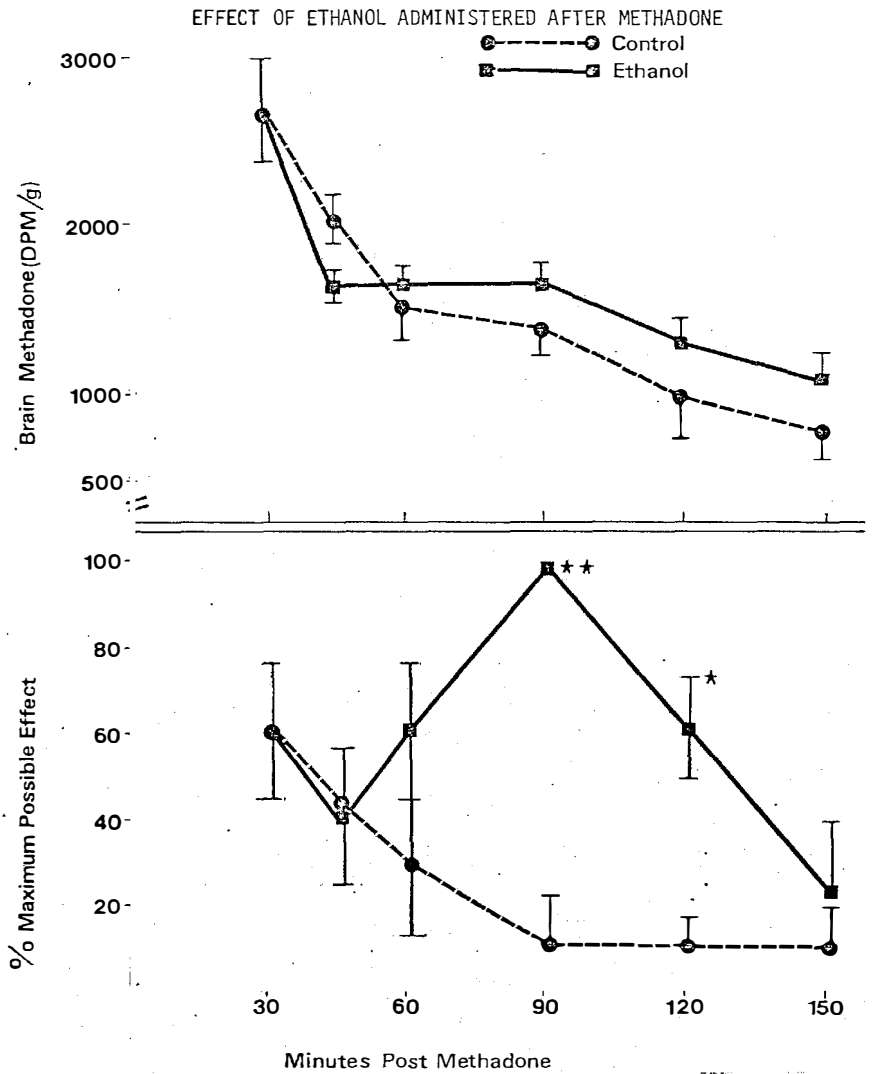
TABLE 8

EFFECT OF ETHANOL ON THE LOSS OF METHADONE FROM THE STOMACH

| Time After Methadone (8 mg/kg, p.o.) | % of Dose in the Stomach | | |
|---|---|---|---|
| | $H_2O$ group | 2.5 g/kg group | 4.0 g/kg group |
| 30 | 9.6 | 22 | 19.6 |
| 60 | - | - | 18.5 |
| 90 | 4.8 | 17 | - |
| 120 | 0.8 | 9.9 | 14.7 |
| 150 | 1.4 | - | 4.8 |

Ethanol was administered (p.o.) 30 minutes prior to methadone.
N = six mice/group.

FIGURE 13




Methadone (8 mg/kg, p.o.) was administered 30 minutes prior to ethanol (2.5 g/kg, p.o.) or water. Each point represents the mean + SEM of six mice per group.

* significantly different from control at $P < .05$

** significantly different from control at $P < .01$

TABLE 9

THE EFFECT OF FOOD DEPRIVATION ON METHADONE ANTINOCICEPTION

| Treatment | $ED_{50}$ | (95% C.I.) |
|---|---|---|
| food and water ad. lib. + water | 3.7 | (2.6 - 5.3) |
| food and water ad. lib. + ethanol | 1.3 | (0.8 - 2.2) |
| food and water ad. lib. + glucose | 3.5 | (2.3 - 5.3) |
| deprived of food + water | 2.8 | (1.8 - 4.5) |
| deprived of food + ethanol | 1.0 | (0.6 - 1.6) |
| deprived of food + glucose | 2.3 | (1.5 - 3.5) |

Ethanol (2.5 g/kg, p.o.) or glucose (5.13 g/kg, p.o.) was simultaneously administered with methadone (s.c.). The $ED_{50}$'s for mice deprived of food with and without ethanol are from figure 11. The methadone + water, and glucose dose-response curves were based on methadone doses of 2,3 and 4 mg/kg (s.c.). The dose response curves with ethanol were based on methadone doses of 0.5, 1, and 2 mg/kg. There were five mice/dose.

antinociception in either free-feeding mice or food-deprived mice. In addition, ethanol produced an approximately equal shift in the $ED_{50}$ of methadone in both free-feeding and food-deprived mice. Methadone seemed to be less potent in free-feeding mice but the $ED_{50}$'s in free-feeding and food-deprived mice were not significantly different.

Thus, since ethanol produced a similar shift in the $ED_{50}$ of methadone in both deprived and free-feeding mice and substitution of isocaloric glucose was without effect in both states, the ethanol-induced alteration of antinociceptive response is not an artifact of food deprivation and furthermore, is not a reflection of the effect of ethanol on the nutritional status of the mice.

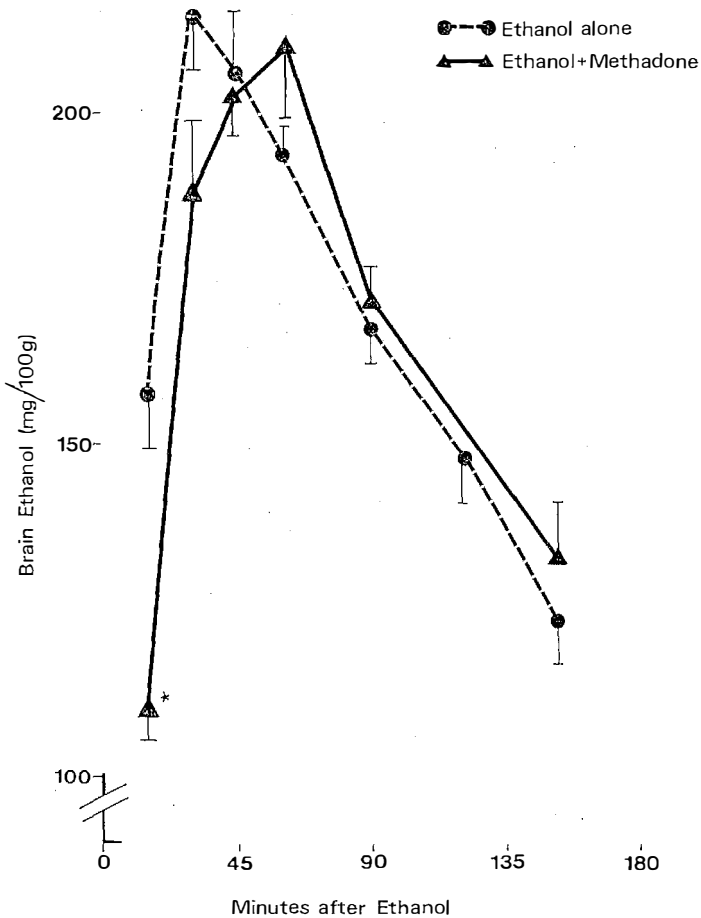
G) Effect of Methadone on the Concentration of Ethanol

Since it was shown in table 2 that high concentrations of ethanol could increase tail-flick latency, another possible mechanism of the observed potentiation involves not the elevation of brain methadone by ethanol, but the elevation of brain ethanol by methadone. The results of brain-ethanol determinations in mice dosed orally with ethanol in combination with either methadone or water are presented in figure 14. Both drugs were given by the same route of administration to increase the possibility for interaction. Table 8 shows that using this dosing schedule at least 10% of the methadone dose was still in the stomach when ethanol was administered.

Since the brain concentration of ethanol when combined with methadone is similar to the ethanol concentration obtained when ethanol is administered alone, and these concentrations of ethanol are without

FIGURE 14.

THE EFFECT OF METHADONE ON BRAIN CONCENTRATION OF ETHANOL




Methadone (8 mg/kg, p.o.) or water was administered 30 minutes prior to ethanol (2.5 g/kg, p.o.). Each point represents the mean ± SEM of at least five mice. Brain ethanol was determined by GLC. * significantly different from ethanol alone at $P < .05$

significant effect on tail-flick latency (table 2), none of the increased antinociceptive effect of ethanol and methadone combinations can be attributed to a simple elevation of the brain concentration of ethanol.

In addition to the data in figure 14, the effect of methadone on ethanol concentrations was also studied in Sprague Dawley rats as shown in table 10. Methadone administered intraperitoneally in doses up to 4.0 mg/kg did not alter the whole brain or whole blood concentrations of ethanol compared to animals that received the same oral dose of ethanol alone. The effect of methadone in this case would be expected to be small since ethanol was administered 45 minutes prior to methadone and animals were sacrificed only 15 minutes after methadone. However, since this is the same dosing schedule used in the dose-response curves presented in figure 6, it shows that the observed increased activity of ethanol and methadone combinations in rats is not due to alterations of brain ethanol levels.

H) Effects of Ethanol on the Excretion of Methadone Metabolites and on the Half-Life of Methadone in Brain

To assess the impact of ethanol administration on the metabolism and excretion of $^{3}$H-methadone, the relative amounts of methadone and its metabolites in bile and urine were determined by TLC analysis. In that the samples were not hydrolyzed prior to solvent extraction, these results deal only with alterations in the amounts of unconjugated metabolites. As can be seen in table 11, the urine, after subcutaneous administration of methadone contains approximately equal concentrations of methadone and EDDP and very little EMDP while bile contains almost equal concentrations of all three substances. When methadone is administered orally

TABLE 10

EFFECT OF METHADONE ON ETHANOL CONCENTRATIONS IN RATS

| Methadone dose mg/kg i.p. | Whole Blood Level mg/100mg ± SEM | Brain Level mg/100g ± SEM |
|---|---|---|
| 0 | 169 ± 59 | 161 ± 38 |
| 2.0 | 177 ± 26 | --- |
| 3.0 | 174 ± 31 | --- |
| 4.0 | 163 ± 36 | 158 ± 27 |

Ethanol (2.5 g/kg, p.o.) was administered 45 minutes prior to Methadone. Levels of Ethanol were determined 15 minutes after Methadone.

TABLE 11

EFFECT OF ETHANOL ON UNCONJUGATED METHADONE METABOLITES IN BILE AND URINE

| Pretreatment | Methadone Administered | URINE | | | BILE | | |
|---|---|---|---|---|---|---|---|
| | | Methadone | EDDP | EMDP | Methadone | EDDP | EMDP |
| Ethanol | s.c. | 44.3 ± 8.1 ** | 53.3 ± 9.1 | 3.1 ± 1.2 | 20.7 ± 3.2 * | 44.8 ± 8.1 | 34.6 ± 6.7 |
| Water | s.c. | 45.7 ± 12 ** | 49.8 ± 12 | 5.2 ± 2.0 | 20.8 ± 11 | 51.8 ± 11 | 27.5 ± 4.6 |
| Ethanol | p.o. | 11.6 ± 2.3 | 86.5 ± 3.2 ** | 2.5 ± 0.6 | 10.2 ± 1.6 | 58.6 ± 5.3 | 31.2 ± 5.1 |
| Water | p.o. | 10.2 ± 1.2 | 84.5 ± 3.7 ** | 5.7 ± 3.1 | 10 ± 2.9 | 64.7 ± 3.9 | 25.1 ± 2.9 |

Values are expressed as percent of total radioactivity present as each metabolite ± SEM. Bile and urine were collected at time of sacrifice 60 to 120 minutes after administration of methadone 8 mg/kg, p.o. or 2 mg/kg, s.c. N = at least 18 mice/treatment. Samples were pooled in groups of three.

* significantly greater than mice dosed by other route of administration at $P < .05$
** significantly greater than mice dosed by other route of administration at $P < .01$

the metabolism of methadone is apparently enhanced as evidenced by the significantly greater relative amount of methadone metabolites after this route of administration.

There was no significant ethanol-induced alteration in the relative proportions of methadone and its metabolites. The observed alteration of metabolites following different routes of administration serves as a positive control and shows that alterations in the rate of metabolism could be detected. Other than EDDP and EMDP, no other $^{3}$H-labeled methadone metabolites were detected in either bile or urine.

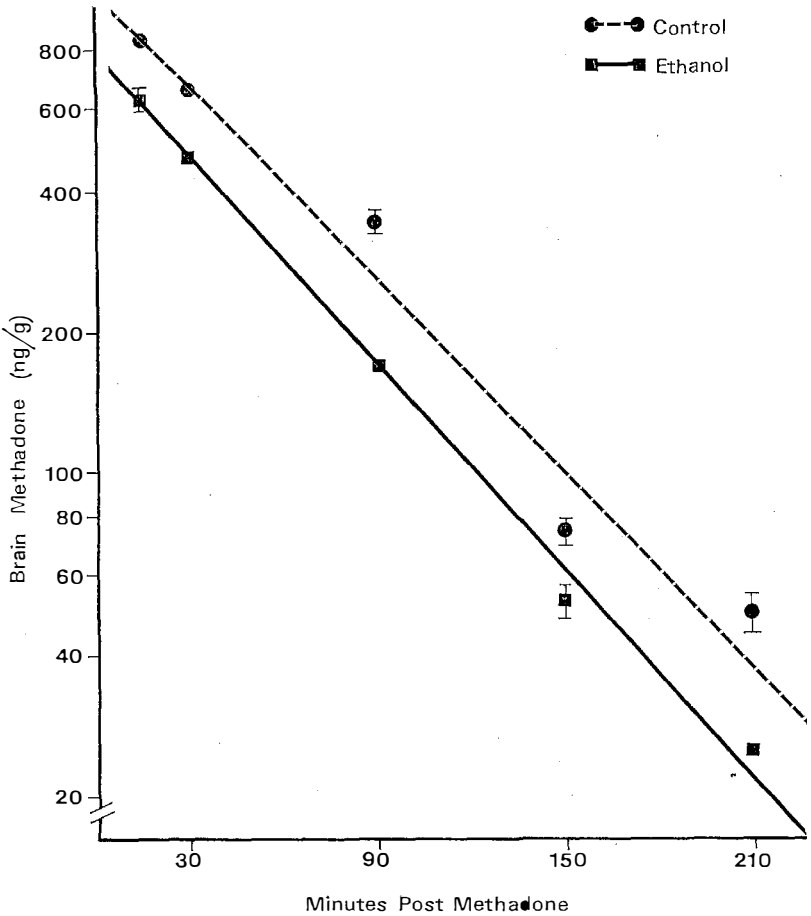
Figure 15 shows that the *in vivo* half-life of methadone in brain is not altered by pretreatment with 2.5 g/kg of ethanol. The half-life in control mice is 45 minutes and in ethanol-pretreated mice is 39 minutes. The brain level data in this figure were taken from figure 8. The dose of methadone was the same in both ethanol and water-pretreated mice but as noted previously, the ethanol-pretreated animals displayed a lower concentration of methadone in brain than the control mice.

In the course of brain and serum $^{3}$H-methadone determinations, the ratio of methadone to total methadone plus metabolites was determined as described in methods. In no experiment was a significant difference in this ratio observed between ethanol and water treated mice (for example, see table 4).

If there were qualitative or quantitative alterations in methadone metabolism these changes would be expected to be most easily observed in the liver where the concentration of the drug is higher and where most

FIGURE 15

WHOLE BRAIN HALF-LIFE OF METHADONE FOLLOWING A 4 mg/kg, s.c. DOSE IN NAIVE MICE




Water or ethanol (2.5 g/kg, p.o.) was administered 30 minutes prior to methadone. Brain levels of methadone were determined by liquid scintillation. Each point represents the mean ± SEM of six mice.

of the metabolism occurs. The results of the analysis of methadone in liver are presented in table 12.

The relative amounts of methadone, EDDP and EMDP are almost identical in animals dosed subcutaneously or orally, with and without ethanol pretreatment. The greater relative amount of EDDP noted in the urine of animals dosed orally is explained by the significantly greater liver/brain ratio in these animals. The $^{3}H$ which was not extracted from the homogenate represents the sum of water soluble metabolites of methadone and any lipid soluble metabolites of methadone not extracted by the solvent. Studies with authentic methadone, EDDP and EMDP indicate that approximately 5% of these compounds are not extracted by the procedure employed. The significantly greater percentage of non-extracable $^{3}H$ in animals dosed orally is probably caused by exchange of label following this route of administration (see appendix I) rather than an increase in the formation of water soluble metabolites.

The routine TLC procedure did not reveal any $^{3}H$ other than that in the zones corresponding to methadone, EDDP and EMDP. To see if other metabolites were formed, the liver samples following oral administration of methadone were further analyzed in two iTLC systems using silica gel impregnated fiberglass sheets as described in methods. Even in these systems no evidence for the formation of additional metabolites in either control or ethanol-pretreated mice could be found. It is possible that other known metabolites of methadone could have been formed but lost in extraction or TLC steps or not detected due to the low specific activity of methadone employed.

Of particular interest are the reduced metabolites of both d and

TABLE 12

EFFECT OF ETHANOL ON METHADONE METABOLITES
IN THE LIVER

| Pretreatment | Methadone Administered | Methadone | EDDP | EMDP | Non-Extractable 3H | Liver/Brain Methadone |
|---|---|---|---|---|---|---|
| Ethanol | s.c. | .38 ± .05 | .34 ± .06 | .14 ± .03 | .15 + .05 | 28.9 + 4.5 |
| Water | s.c. | .35 ± .06 | .33 ± .09 | .13 ± .03 | .19 ± .04 | 21.0 ± 6.9 |
| Ethanol | p.o. | .30 ± .03 | .29 ± .06 | .10 ± .02 | .31 ± .05 | 62.7 ± 10.7 ** |
| Water | p.o. | .28 ± .07 | .25 ± .05 | .12 + .06 | .35 ± .07 | 55.5 ± 7.1 ** |

Results are expressed as the ratio of methadone or metabolite DPM to the total DPM/g of liver (± SEM) 60 minutes after the methadone dose as described in Methods. Ethanol or water administered p.o. 30 minutes prior to methadone (8 mg/kg, p.o.) or simultaneously with methadone (2 mg/kg, s.c.). N= 12 mice/group.

** significantly different from mice dosed s.c. at $P < .01$

l-methadone, alpha-l-methadol and alpha-d-methadol (152) respectively. Although alpha-d-methadol is not an active metabolite, alpha-l-methadol possess antinociceptive activity of the same order of magnitude as d,l-methadone (152). Since the administration of ethanol produces a marked increase in the NADH/NAD ratio in the liver (87), it is possible ethanol would also increase the production of these reduced metabolites.

I) The Effect of Ethanol on the Antinociceptive Activity of l-Methadone and Other Narcotics

Rather than go through an extensive search for the reduced metabolites of methadone, their possible role in the ethanol-mediated potentiation of methadone antinociception was evaluated by combining ethanol and l-methadone. If increased production of reduced metabolites of methadone is a reason for increased antinociception from d,l-methadone then ethanol would be unable to potentiate the action of l-methadone since its reduction metabolite possesses almost no antinociceptive activity.

The results of this experiment are presented in figure 16. As would be expected, the control $ED_{50}$ of l-methadone is approximately one-half that of d, l-methadone. More interestingly, simultaneous administration of 2.5 g/kg of ethanol produced a significant potentiation of l-methadone antinociception, reducing the $ED_{50}$ from 1.4 mg/kg to 0.6 mg/kg.

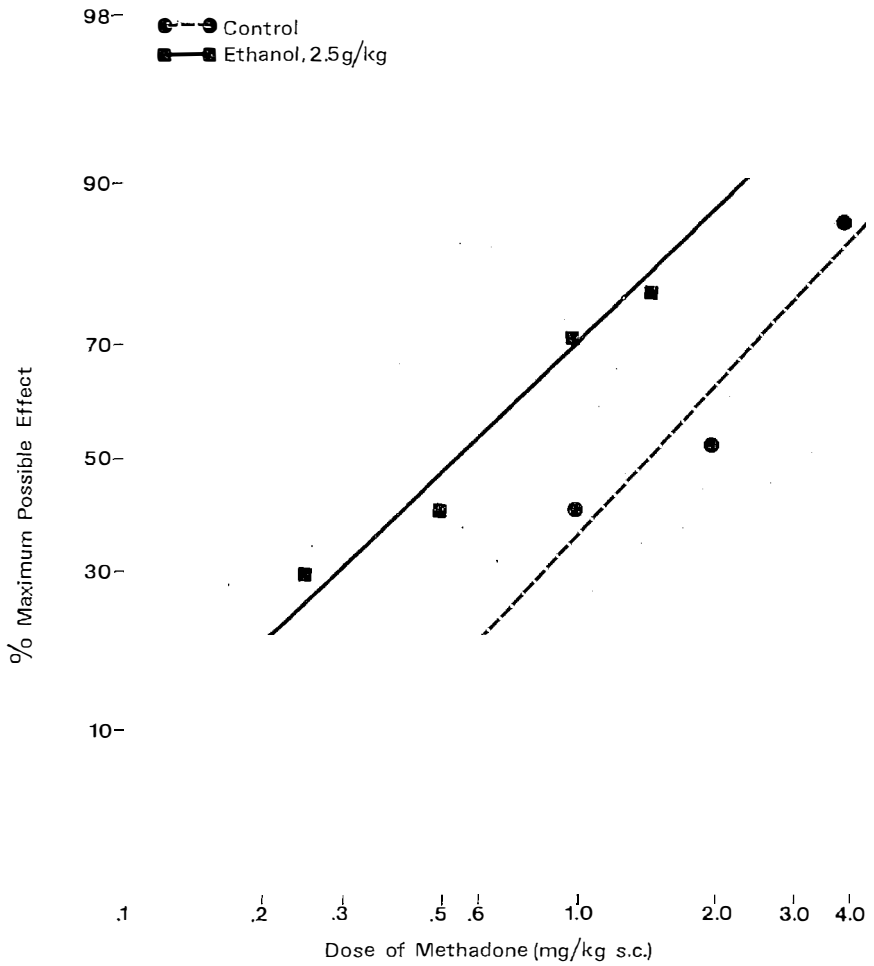
The results of combination of ethanol and other narcotics are presented in Appendix II.

J) Antagonism of the Tail-Flick Activity of Ethanol and Methadone by Naloxone

If the increased antinociceptive effectiveness of combinations of methadone and ethanol is due to a true potentiation of the narcotic

FIGURE 16

THE EFFECT OF ETHANOL PRETREATMENT ON THE TAIL-FLICK ACTIVITY OF l-METHADONE




Ethanol (2.5 g/kg, p.o.) or water was coadministered with methadone and animals were tested 60 minutes later.. N = at least six mice/group

Control $ED_{50}$= 1.4 (0.9-2.2), with ethanol $ED_{50}$= 0.6 (0.4-0.9)

agonist properties of methadone by ethanol then this effect should be antagonized by a specific narcotic antagonist such as naloxone. To test this, 36 mice were treated with 2.5 g/kg ethanol 45 minutes prior to subcutaneous administration of 1.8 mg/kg methadone. Another group of mice was pretreated with water and administered 3.6 mg/kg methadone subcutaneously. Twelve mice from each group were tested for tail-flick latency 15 minutes after methadone. These mice also received subcutaneous saline 30 minutes prior to testing. The mice treated with ethanol and methadone had an average concentration of methadone in the brain of 245 ng/g $\pm$ 32 and an antinociceptive effect of 74% MPE. The animals which received 3.6 mg/kg methadone had an average concentration of methadone in the brain of 740 ng/g $\pm$ 27 and an antinociceptive effect of 86% MPE.

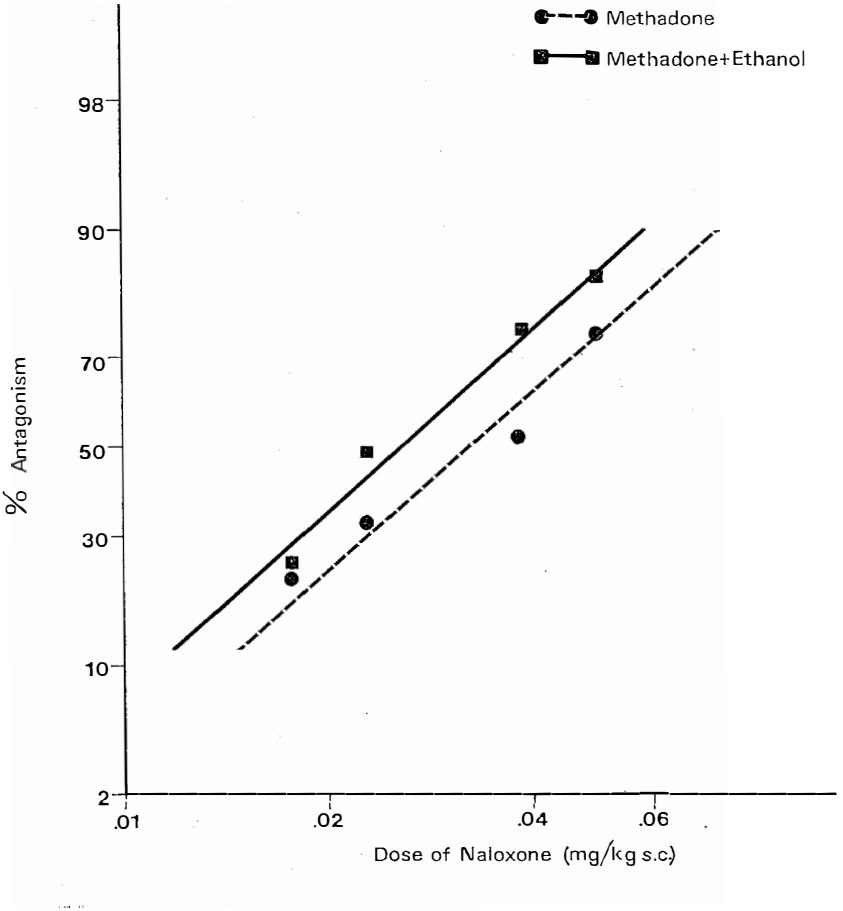
The remaining mice were administered various doses of naloxone instead of saline and tested for tail-flick latency in order to establish the dose-response curves presented in figure 17. The mice which received 0.05 mg/kg naloxone had an average brain-methadone level of 276 ng/g $\pm$ 19 and 702 ng/g $\pm$ 37 in the ethanol and methadone, and methadone alone groups respectively.

To assess the effect, if any, of naloxone on the tail-flick activity of ethanol, the highest dose of naloxone used, 0.05 mg/kg was combined with high doses of ethanol (table 13). Although 6.0 g/kg ethanol produced a significant elevation of tail-flick latency, this effect was not altered by naloxone.

Despite the lack of effect of 0.05 mg/kg naloxone on ethanol alone, naloxone at this dose was able to produce an almost 90% reduction in the

FIGURE 17

ANTAGONISM OF THE TAIL-FLICK ACTIVITY OF ETHANOL AND METHADONE BY NALOXONE




Animals were pretreated with either ethanol (2.5 g/kg, p.o.) or water at Time = 0, injected with naloxone at T=30 min., injected s.c. with 1.8 or 3.6 mg/kg methadone respectively at T=45 min. and tested at T=60 min. N = 6 mice/group.

$ID_{50}$ against Methadone = 0.032 mg/kg (.021 - .049)
$ID_{50}$ against Ethanol and Methadone = 0.026 mg/kg (.019 - .035)

TABLE 13

EFFECT OF NALOXONE ON THE TAIL-FLICK ACTIVITY OF ETHANOL

| Dose of Ethanol g/kg, p.o. | Brain Level of Ethanol mg/100 g ± SEM | %MPE with saline | %MPE with 0.05 mg/kg Naloxone s.c. |
|---|---|---|---|
| 2.5 | 193 ± 7 | 2.0 | - |
| 4.0 | 312 ± 25 | -4.1 | 1.2 |
| 6.0 | 500 ± 45 | 85 | 78 |

Mice were administered ethanol 30 minutes prior to naloxone and tested 30 minutes later. There were five animals/group.

antinociceptive effect of ethanol and methadone. In addition, as shown in figure 17, naloxone antagonized ethanol and methadone in the same manner as it antagonized methadone alone. That is, although the mice administered ethanol and methadone had much lower brain-methadone levels compared to the mice administered methadone alone, the slopes of the dose-response curves as well as the calculated $ID_{50}$'s were identical in both groups.

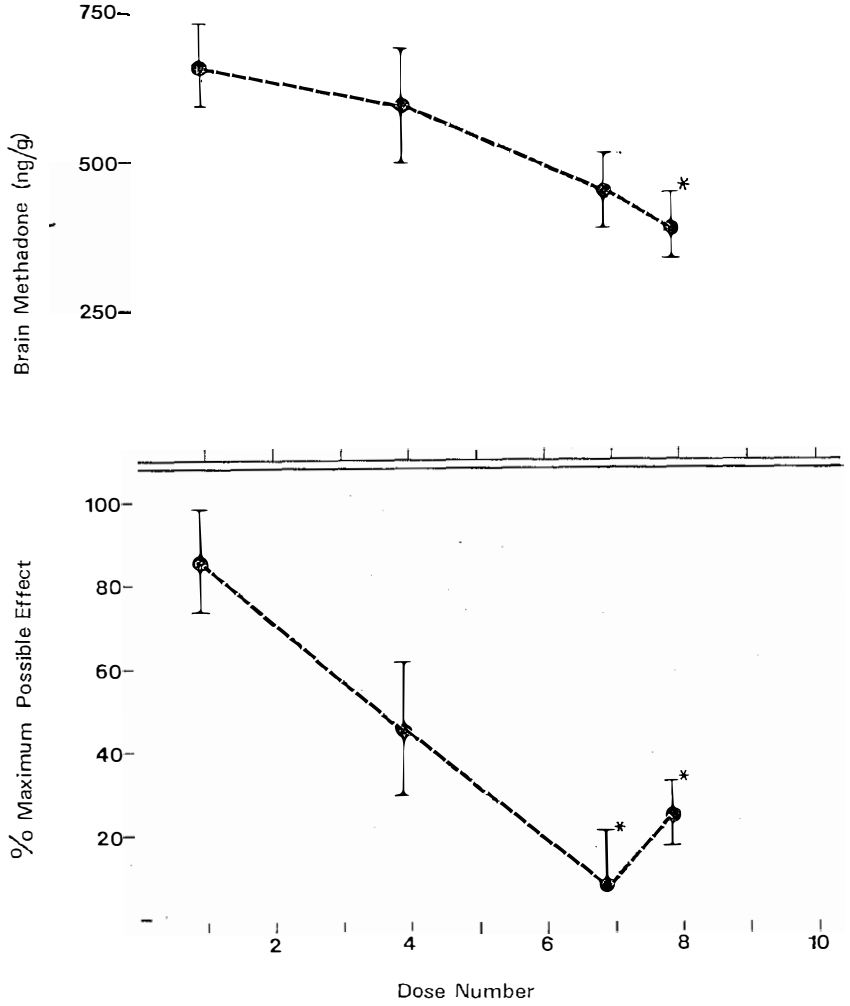
K) Effects of Ethanol in Methadone-Tolerant Mice

Since the combined use of ethanol and methadone in humans most frequently occurs in methadone maintenance patients who are tolerant to methadone it is important to investigate the effects of the combination in a chronic as well as in an acute situation. To do this, mice were made tolerant to methadone by once daily administration of methadone at a constant oral dose of 20 mg/kg.

Since the half-life of methadone in the brain of mice following subcutaneous administration is less than 1 hour it was thought that the dose would have to be increased or administered more frequently to produce tolerance. To monitor tolerance, groups of six mice were tested for tail-flick activity and brain concentration of methadone during the course of the 20 mg/kg/day treatment. As shown in figure 18, after four doses of methadone the antinociceptive activity of methadone was reduced by half although brain methadone was only slightly decreased compared to the results of the first dose. It was therefore thought that tolerance would develop even at a daily dose of 20 mg/kg and this dose was continued. Figure 18 shows that after 8 days (or eight 20 mg/kg doses) the brain concentration of methadone 60 minutes after a 20 mg/kg dose

FIGURE 18

DEVELOPMENT OF TOLERANCE TO DAILY DOSES OF METHADONE
20 mg/kg/day (p.o.)




Tail Flick activity was determined and animals were sacrificed 60 min after the daily dose of methadone. Brain methadone was determined by GLC. Each point represents the mean ± SEM of six mice.

* significantly different from result of first dose at $P < .05$

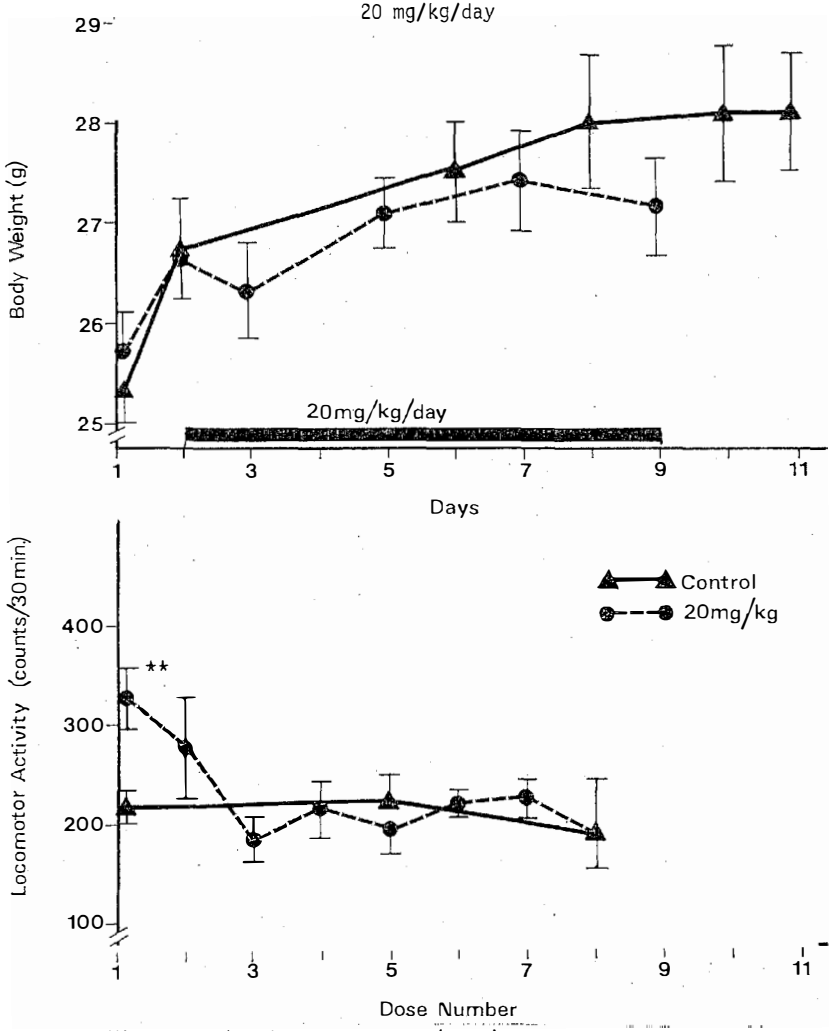
had significantly declined from 650 ng/g to 400 ng/g and the antinociceptive effect had declined from 85% MPE to 25% MPE. Thus, chronic methadone administration had apparently induced its own metabolism but the fact that the antinociceptive effect declined more rapidly than the brain concentration of methadone indicates some CNS tolerance to methadone. A parallel group of twelve mice were tested for physical dependence 24 hours after their eighth dose of methadone (20 mg/kg/day). Subcutaneous injection of naloxone at 3 or 9 mg/kg did not precipitate any jumping in these mice. Naloxone did produce signs of hyperventilation and increased urination and defecation. Analysis of the brains from these mice by GC/MS failed to reveal any residual methadone. Considering the sensitivity of the GC/MS assay, this indicated that if any free methadone existed in the brain at this time it was at a level less than 5 ng/g.

The effect of the daily administration of methadone at a dose of 20 mg/kg on body weight and locomotor activity was also determined. As can be seen in figure 19, the first dose of methadone produced a significant elevation in locomotor activity. Tolerance to this effect was rapid and by the third dose there was no elevation in locomotor activity compared to control animals. The methadone treatment produced some decline in body weight but the alteration was not significant.

Since the animals maintained on methadone had free access to food and water at all times, the absorption and antinociceptive activity of an oral dose of methadone in free-feeding naive mice was investigated. Figure 20 shows the whole brain concentrations of methadone and the antinociceptive effect of methadone in these animals. Animals tested and

FIGURE 19

THE EFFECT OF METHADONE ON BODY WEIGHT AND LOCOMOTOR ACTIVITY
20 mg/kg/day

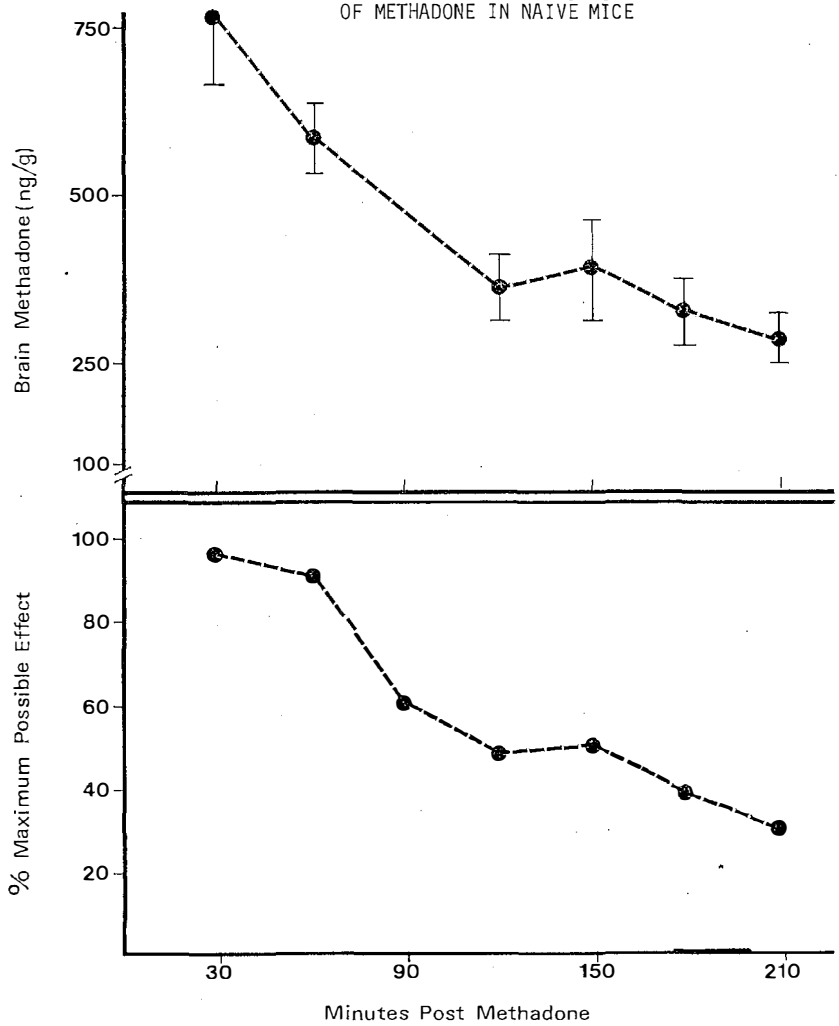



Mice were dosed once per day (p.o.). Locomotor activity was measured on at least 12 pairs of mice from 30 to 60 min. after the daily dose.

** significantly different from control at $P < .01$

FIGURE 20.

THE EFFECT OF A SINGLE 20 mg/kg ORAL DOSE OF METHADONE IN NAIVE MICE




Each point represents the mean of six mice ± SEM. Brain levels were determined by GLC.

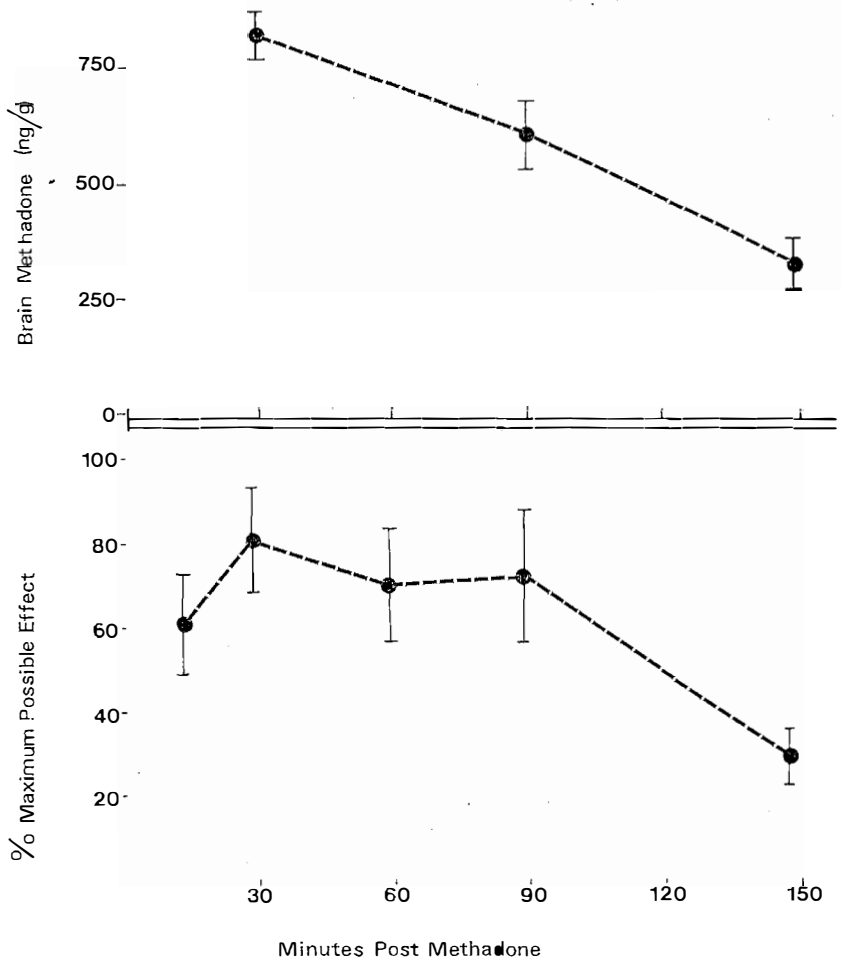
sacrificed at 210 minutes were also tested at 90 minutes post methadone.

In order to investigate whether the tolerant mice would still display an increased antinociceptive effect if the brain concentration of methadone was increased, mice maintained on 20 mg/kg/day were administered 40 mg/kg methadone (figure 21). This dose of methadone in tolerant mice produced a brain concentration of methadone almost equal to the concentration obtained following a 20 mg/kg dose in naive mice and greater than that following the normal maintenance dose of 20 mg/kg. The tolerant mice did derive a greater antinociceptive effect from these increased methadone concentrations since at 60 minutes these mice displayed 70% MPE while mice maintained on 20 mg/kg displayed 25% MPE. Mice tested at 15 and 60 minutes were retested and sacrificed at 150 and 90 minutes respectively.

The effect of water or 2.5 g/kg ethanol administered 30 minutes before the eighth methadone dose of 20 mg/kg is presented in figure 22. At 30 minutes both groups had identical brain concentrations of methadone. At later time periods the ethanol-pretreated mice had brain concentrations of methadone greater than control. To compare the effects of ethanol on brain methadone and antinociception, the areas under the corresponding curves were integrated from 30 to 150 minutes post methadone (table 14). These results indicate that although ethanol pretreatment in tolerant mice produced a 35% greater brain level, it increased the antinociceptive response by 367%. A 40 mg/kg dose increased brain methadone by 70% compared to 20 mg/kg with ethanol in tolerant mice but still produced an antinociceptive response 13% less. Thus, although ethanol in free-feeding, methadone-tolerant mice produced slightly greater brain

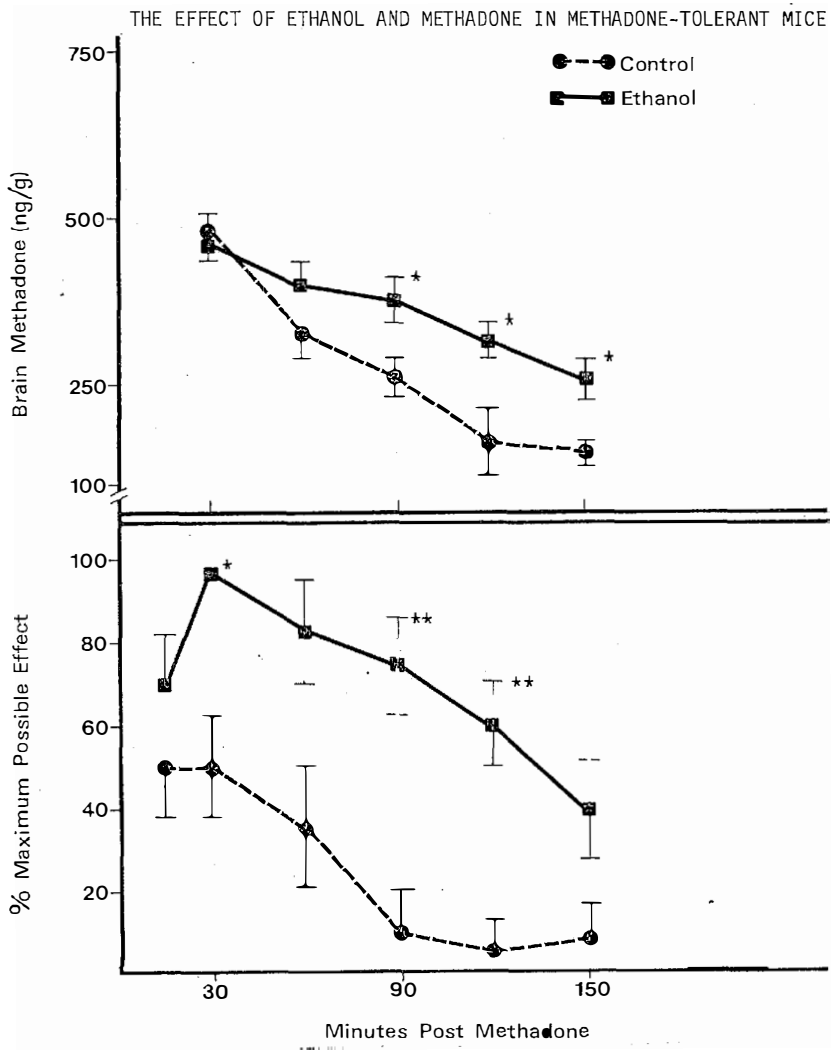
FIGURE 21

TAIL-FLICK ACTIVITY AND BRAIN-METHADONE LEVELS IN TOLERANT MICE (40 mg/kg, p.o.)




Mice were administered methadone (20 mg/kg/day, p.o.) for seven previous days. The eighth dose was 40 mg/kg, p.o. Brain levels of methadone were determined by GLC. Each point represents the mean ± SEM of six mice.

FIGURE 22




Animals were administered methadone (20 mg/kg/day, p.o.) for seven previous days. Thirty min. before the eighth dose mice were dosed with ethanol(2.5 g/kg, p.o.) or water. Each point represents the mean + SEM of six mice. Brain levels determined by GLC.

* significantly different from control at $P < .05$
** significantly different from control at $P < .01$

TABLE 14

COMPARISON OF AREAS UNDER GRAPHS OF METHADONE CONCENTRATION VS TIME AND % MPE VS TIME

| Treatment | Figure # | Response vs Time | Brain Level vs Time |
|---|---|---|---|
| 20 mg/kg, tolerant, ETOH | 22 | 367 | 135 |
| 20 mg/kg, tolerant, water | 22 | (100) | (100) |
| 20 mg/kg, naive, water | 20 | 343 | 194 |
| 40 mg/kg, tolerant, water | 21 | 320 | 235 |

Areas were integrated from 30 to 150 minutes post methadone and normalized against the areas under the curves from the 20 mg/kg tolerant mice administered water.

concentrations of methadone compared to controls, ethanol was still able to potentiate methadone in the tolerant state as evidenced by the much greater increase in antinociceptive response. Mice tested at 15 minutes post methadone were retested and sacrificed at 150 minutes.

As shown in figure 23, the half-life of methadone in brain after a 20 mg/kg oral dose in free-feeding naive mice is 150 minutes. In tolerant, free-feeding animals the half-life is decreased to 66 minutes in control mice but ethanol pretreatment in tolerant mice increased the half-life to 144 minutes.

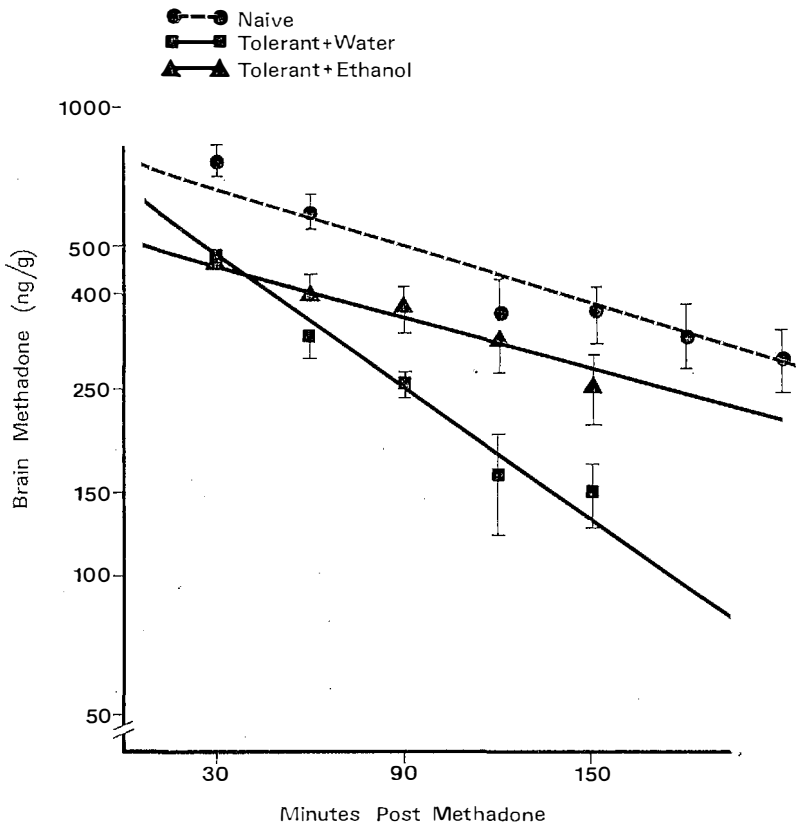
Analysis of the serum concentration of ethanol in tolerant mice (table 15) shows that the initial absorption of ethanol is slightly decreased compared to naive mice deprived of food. At later times the tolerant mice had slightly greater serum concentrations of ethanol. Comparison of serum concentrations of ethanol in tolerant free-feeding mice and naive free-feeding mice shows that these alterations are probably due to the presence of food in the stomach rather than to tolerance to methadone. The ethanol concentration obtained in free-feeding tolerant mice is not significantly different from that obtained in food-deprived mice.

L) Toxic Effects of Ethanol and Methadone Combinations

Since the antinociceptive properties of methadone were potentiated by ethanol, an investigation of the effect of ethanol on the lethal properties of methadone was undertaken to see if this effect would also be potentiated. Animals were deprived of food for the 16 hours prior to dosing. The $LD_{50}$ experiment was started at 4:00 p.m.

FIGURE 23

WHOLE BRAIN HALFLIFE OF METHADONE FOLLOWING A 20 mg/kg ORAL DOSE IN NAIVE AND TOLERENT MICE




Mice were administered methadone (20 mg/kg/day, p.o.) for seven previous days. Thirty min. before the eighth dose mice were dosed with ethanol(2.5 g/kg, p.o) or water. Brain methadone was determined by GLC. Each point represents the mean ± SEM of six mice.

TABLE 15

ABSORPTION OF ETHANOL IN METHADONE-TOLERENT MICE
WITH FOOD AND WATER AD. LIB.

| Time After Ethanol 2.5 g/kg p.o. (min) | Serum Ethanol (mg/100 ml ± SEM) | | |
|---|---|---|---|
| | Naive* | Naive** | Methadone Tolerent |
| 60 | 222 ± 40 | --- | 174 ± 21 |
| 90 | 198 ± 21 | 226 ± 17 | 203 ± 31 |
| 120 | 167 ± 29 | 161 ± 10 | 153 ± 15 |
| 150 | 140 ± 19 | 155 ± 12 | 162 ± 25 |

*Deprived of food for 16 hours prior to dosing

**With free access to food and water

Figure 24 shows the effect of orally administered ethanol on the $LD_{50}$ of orally administered methadone. All animals that died appeared to die as a result of respiratory depression. No convulsions were observed. The high doses of methadone employed increased the locomotor activity of control mice and seemed to counteract the initially decreased locomotor and exploratory behavior of the ethanol-pretreated mice. Although not initially anticipated, one of the most significant effects of ethanol in this experiment was an alteration in the time of death.

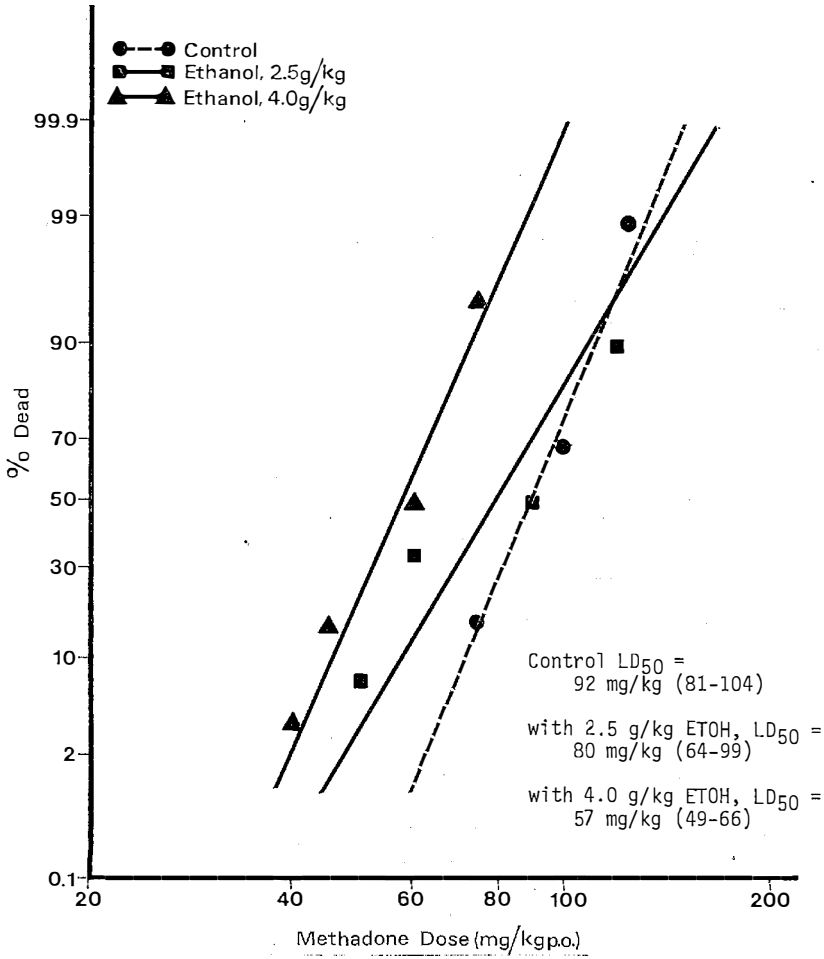
Control animals died at an average of 19.7 $\pm$ 3.5 minutes after methadone administration. Pretreatment with 2.5 g/kg ethanol increased the latency of death to 36.9 $\pm$ 11.3 minutes. The mice pretreated with 4 g/kg that died within the 6 hour observation period died at an average of 98 $\pm$ 17 minutes after dosing. All survivors were allowed access to food and water 6 hours after dosing. The only deaths between 6 hours and 24 hours were several of the animals pretreated with 4 g/kg. Although included in the calculation of the 24 hour $LD_{50}$, these animals were excluded from the calculation of latency of death and brain concentration of methadone at death.

The 24 hour $LD_{50}$ of methadone was not significantly altered by pretreatment with 2.5 g/kg ethanol. Pretreatment with 4 g/kg ethanol did result in a significant decrease in the $LD_{50}$ of methadone.

Because of the previously demonstrated effects of ethanol on oral absorption of methadone (figure 12) and the above noted alterations in latency of death, the $LD_{50}$ experiment was repeated using subcutaneous administration of methadone as shown in figure 25. In this case, the

FIGURE 24

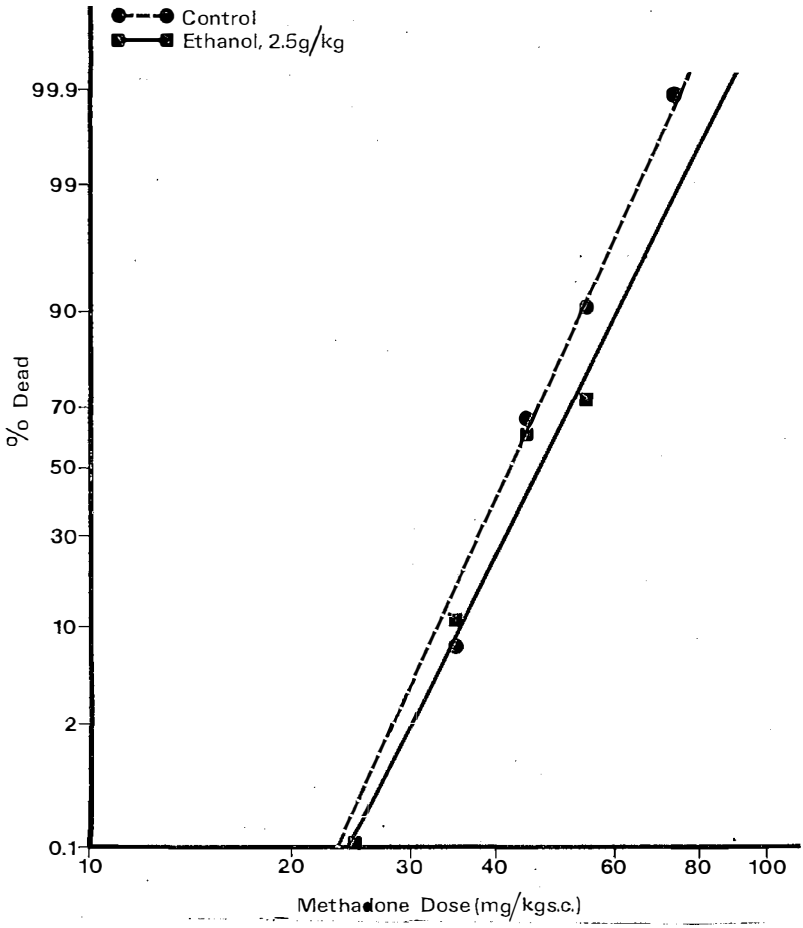
THE EFFECT OF ETHANOL ON THE $LD_{50}$ OF METHADONE (p.o.)




Mice were pretreated with either ethanol (2.5 or 4.0 g/kg, p.o.) or water 30 min. prior to administration of methadone. Mice were observed for 24 hrs. after the dose. There were at least six mice per group.

FIGURE 25

THE EFFECT OF ETHANOL ON THE $LD_{50}$ OF METHADONE (s.c.)




Mice were pretreated with either ethanol (2.5 g/kg, p.o.) or water 30 minutes prior to administration of methadone. Mice were observed for 24 hrs. after the dose. There were 10 mice per group.

Control $LD_{50}$ = 41 mg/kg (33-51)

with 2.5 g/kg ETOH $LD_{50}$ = 49 mg/kg (44-51)

latency of death was equal in both ethanol and water-pretreated animals at 14.2 ± 7 and 16.3 ± 9 minutes respectively. As noted following oral administration, the subcutaneous $LD_{50}$ of methadone was also not altered by pretreatment with 2.5 g/kg ethanol.

The brain concentrations of methadone in animals that died during the course of the $LD_{50}$ experiments were determined by GLC. As shown in table 16, animals pretreated with 2.5 g/kg in the oral $LD_{50}$ experiment died at significantly lower brain concentrations of methadone than controls. The animals pretreated with 4.0 g/kg died at even lower brain concentrations of methadone.

In parallel with the daily administration of methadone at 20 mg/kg/day to produce tolerance, another group of mice were administered increasing oral doses of methadone until they were being maintained on 100 mg/kg/day. In these mice there were no deaths due to the daily dose when given alone. However, when these mice were administered either 200 mg/kg methadone or 2.5 g/kg ethanol combined with the normal daily dose of 100 mg/kg methadone, approximately 30% of each group of mice died (table 16). Analysis of brain methadone in these animals showed that the animals that died as a result of ethanol and methadone administration died at slightly lower brain methadone levels than the animals which died due to methadone alone.

Prompted by this observation of an approximate twofold increase in toxicity in animals maintained on high doses of methadone (table 16), the $LD_{50}$ of ethanol in both naive and tolerant mice was investigated as shown in figure 26 and table 17.

TABLE 16

BRAIN CONCENTRATIONS OF METHADONE IN METHADONE DEATHS

| | ETHANOL DOSE | METHADONE DOSES | BRAIN METHADONE LEVEL AT DEATH, μg/g ± SEM |
|---|---|---|---|
| Acute (p.o.) | 0.0 | 75-120 mg/kg | 4.9 ± 0.4 |
| | 2.5 | 75-120 mg/kg | 3.5 ± 0.5* |
| | 4.0 | 65-90 mg/kg | 1.9 ± 0.3 *** |
| Acute (s.c.) | 0.0 | 45-55 mg/kg | 5.5 ± 0.5 |
| | 2.5 | 45-55 mg/kg | 3.9 ± 0.3 * |
| Tolerant [a] (p.o.) | 0.0 | 100 mg/kg | |
| | 2.5 | 100 mg/kg [b] | 5.1 ± 0.7 |
| | 0.0 | 200 mg/kg [c] | 7.6 ± 1.2 |

[a]Tolerant animals were administered methadone, 100 mg/kg/day for seven previous days. This dosing level was attained by first administering 50 mg/kg for one day, then 75 mg/kg for one day and finally 100 mg/kg.

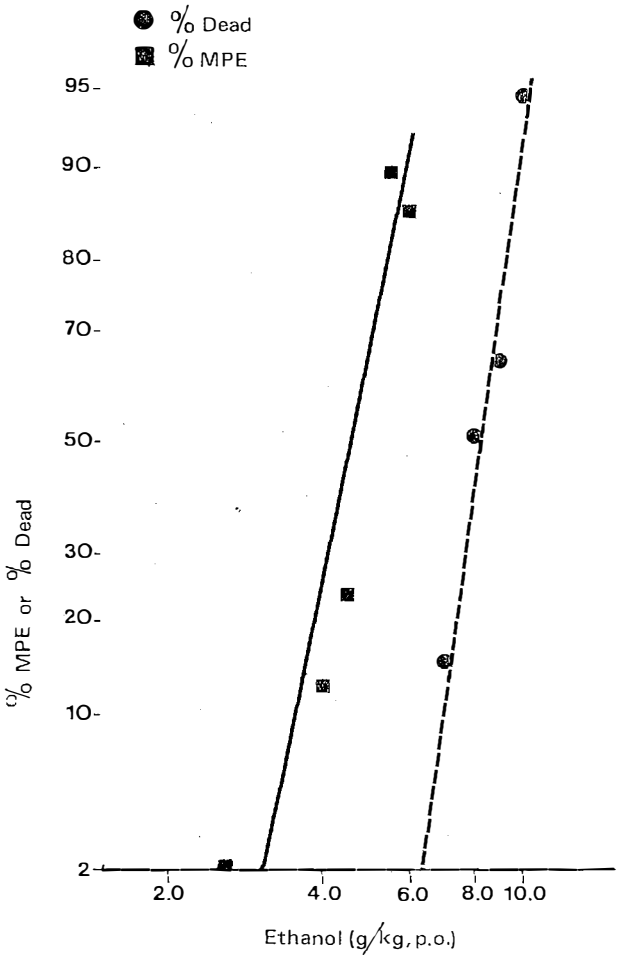
[b]2.5 g/kg ethanol with 100 mg/kg as the eighth dose killed 9/30 mice (=30%).

[c]Water with 200 mg/kg as the eighth dose killed 5/18 mice (=28%).

* significantly different from control at $P < .05$
*** significantly different from control at $P < .005$

FIGURE 26

$ED_{50}$ AND $LD_{50}$ OF ETHANOL IN MICE




$ED_{50}$ =4.9 g/kg (4.1-5.8) $LD_{50}$ = 8.3 g/kg (7.2-9.5)

There were at least six mice/group. Tail flick activity was determined 60 min. after ethanol.

TABLE 17

$LD_{50}$ OF ETHANOL IN METHADONE-TOLERANT MICE

| Treatment | $LD_{50}$ g/kg, p.o. | (95% C.I.) |
|---|---|---|
| Naive | 8.3 | (7.2-9.5) |
| 40 mg/kg/day | 7.0 | (5.8-8.4) |
| 100 mg/kg/day | 2.8* | (2.3-3.4) |

Mice were not deprived of food and were administered ethanol as a 25% w/v solution 30 minutes prior to their daily dose of methadone. There were four groups of six mice/group in each treatment. Mice in the 40 mg/kg/day group were administered 40 mg/kg/day for ten days. Mice in the 100 mg/kg/day group were dosed as described in table 16.

*Significantly different from naive mice @ P <.05

# DISCUSSION

## A) Antinociceptive Activity of Ethanol and Methadone

The results of this study demonstrate that a 2.5 g/kg oral dose of ethanol did not significantly alter the hot-plate response of mice, although a dose of 4 g/kg interfered with this response. This finding confirms and extends the early work of Smith and Loomis (144) who found that the intravenous administration of ethanol at a dose of 1.0 g/kg did not alter the latency of mice in the hot-plate procedure.

The present study also shows that oral doses of ethanol less than 4.5 g/kg did not significantly alter the tail-flick response of rats. Administration of a 2.5 g/kg oral dose of ethanol to mice did not alter their tail-flick response at any time period examined. Increasing the dose to 4.0 g/kg produced a slight increase in latency, but significant effects on tail-flick response were not observed until higher doses were administered. As shown in figure 26, the slope of the $ED_{50}$ and $LD_{50}$ curves are similar, which suggests the observed alterations in tail-flick latency may be due to general CNS depression.

These results are similar to the results of other tests of antinociception, which are also unaffected by low doses of ethanol. For example, a 2.0 g/kg oral dose of ethanol did not significantly alter the p-phenylquinone writhing response (65, 143), however the effects of higher doses were not reported. Using the toothpulp threshold of rabbits, Stanton and Keasling (149) showed that intravenous doses of ethanol less than 3 g/kg did not produce an effect, but increasing the dose of ethanol produced elevations in threshold which were well correlated with

blood-ethanol levels. In contrast to these results, some test procedures such as shock titration procedures in trained rats or electrical stimulation of the rat tail are altered by intraperitoneal administration of as little as 1.0 g/kg ethanol (22).

The effect of ethanol is similar to that of chloral hydrate and paraldehyde, which also display antinociceptive properties at high doses in the electrical stimulation of the mouse-tail test (112). On the other hand, barbiturates have been reported to have either no antinociceptive properties in the rat-tail compression test (52) or to produce increased sensivity to pain (hyperalgesia) at subanesthetic doses in the hot-plate (144) and electrical stimulation of the mouse-tail tests (112).

In this study the effect of ethanol on methadone antinociception has been studied in a number of different experimental protocols. When a dose of ethanol not active by itself in the tail-flick test (2.5 g/kg) was administered prior to (figure 8) or simultaneously with (figure 9) subcutaneously administered methadone, the antinociceptive response was greater than methadone treated controls at every time point studied. The same was true when both drugs were administered by the oral route (figure 12). This increased antinociceptive effect is not due to an ethanol-mediated increase in whole brain concentrations of methadone. In fact, ethanol pretreatment, especially when methadone is also administered orally, results in significant decreases in the whole brain concentration of methadone when compared to water-pretreated controls. However, ethanol increased the antinociceptive effect of methadone to such a degree that in spite of the lower brain concentrations of methadone, ethanol-pretreated animals displayed a greater antinociceptive

effect.

To reliably determine the magnitude and significance of the differences between ethanol and water-treated animals, dose-response curves were established at various times after administration of methadone. A summary of the dose-response curves is presented in table 18. These results show that a dose of ethanol which by itself is not active in the tail-flick or hot-plate tests produced a significant increase in the antinociceptive effect of methadone in both rats and mice as measured by the tail-flick test, and also increased methadone antinociception as measured in the mouse hot-plate test. The potency ratio when mice were administered ethanol and methadone simultaneously and tested at 60 minutes post methadone is slightly greater than the potency ratio when mice were administered ethanol 45 minutes prior to methadone and tested 15 minutes post methadone. As can be seen in tables 3 and 6, pretreatment with ethanol lowered brain methadone compared to controls while the brain concentration of methadone was not decreased relative to controls when methadone was coadministered with ethanol, which may be responsible for the difference in the potency ratios.

Due to the ability of ethanol pretreatment to decrease brain concentrations of methadone, a more useful measure of relative potency may be obtained from a brain-methadone concentration vs response curve (figure 7). When examined in this way, it can be seen that ethanol increased methadone antinociception to the same degree at 15 minutes post methadone as it did at 60 minutes post methadone.

The calculated whole brain $EC_{50}$ of methadone (360 ng/g) was approximately four times the whole brain $EC_{50}$ of morphine in the tail-flick

TABLE 18

SUMMARY OF DOSE-RESPONSE EXPERIMENTS

| Dosing Protocol | Species | Test[a] | Figure # | Potency Ratio[b] |
|---|---|---|---|---|
| $H_2O$/E,45,M@15 | Mouse | HP | 4 | 2.0 |
| $H_2O$/E,45,M@15 | Mouse | TF | 5 | 2.5[c] |
| $H_2O$/E,45,M@15 | Rat | TF | 6 | 2.0 |
| $H_2O$/E and M@60 | Mouse | TF | 11 | 2.8 |

A dosing protocol where water or ethanol (2.5 g/kg, p.o.) was administered 45 minutes prior to methadone (s.c.) and animals were tested 15 minutes later is abbreviated as $H_2O$/E,45,M@15.

[a]HP = hot-plate test, TF = tail-flick test.

[b]Potency ratio = control $ED_{50}/ED_{50}$ in presence of ethanol. In each case the $ED_{50}$'s are significantly different at the 95% confidence level.

[c]Pretreatment with 4.0 g/kg ethanol gave a potency ratio of 10.

test (117). Studies of the relative affinity of methadone and morphine for stereospecific binding sites (119) as well as the relative potency of both drugs after intraventricular injection (86) indicate that methadone is intrinisically less effective as an analgesic than morphine. The observation from this study that greater whole brain concentrations of methadone are required to produce the same antinociceptive response as a lesser whole brain concentration of morphine is in agreement with these studies.

The dose-response curves for methadone in the presence of 0, 2.5 or 4.0 g/kg ethanol (figure 5) clearly show that the effect of ethanol on methadone antinociception is dependent upon the dose of ethanol, since the $ED_{50}$ of methadone in the presence of 4 g/kg ethanol is significantly different from the $ED_{50}$ of methadone in the presence of 2.5 g/kg ethanol, which is significantly different from the control $ED_{50}$ of methadone.

In addition to being responsive to different doses of ethanol at a fixed time after ethanol, the increased antinociception also seems to be responsive to changes in brain ethanol at various times after a dose of ethanol. Dose-response curves to methadone were not established at various times after a single dose of ethanol. However, the results in figure 13 where ethanol was administered 30 minutes after methadone show that the amount of methadone in brain is almost constant from 15 minutes post ethanol (i.e. 45 minutes post methadone) to 60 minutes post ethanol, however, the antinociceptive response increased from 40% MPE to 100% MPE as brain ethanol levels (from figure 14) rose from 116 to 208 mg/100 g. At 30 minutes post ethanol, the brain concentration

of ethanol was almost at its peak value, but the antinociceptive response was only 60% MPE. At 60 minutes post ethanol, the brain concentration of ethanol was slightly greater than at 30 minutes, but the antinociceptive response increased from 60 to 100% MPE. Thus it seems that increases in antinociceptive response lagged slightly behind increases in brain concentrations of ethanol. The results of administration of ethanol 90 minutes prior to methadone compared to simultaneous administration of ethanol and methadone (table 7) suggest that the decay of the increased antinociception parallels the decline in brain ethanol.

In addition to experiments in naive mice, the effect of ethanol on methadone antinociception in methadone-tolerant mice was also examined (figure 22). In this experiment ethanol-treated mice had higher concentrations of methadone in the brain than controls, although the observed increase in antinociceptive effect was still greater than would be expected solely from the increase in brain methadone (table 14). Since ethanol produced significant alterations in the brain concentration of methadone in both naive and tolerant mice, it was desirable to compare the degree of potentiation after removing the influence of alterations in brain concentrations of methadone. This was accomplished by integrating the areas under the brain-level curve vs. time and the antinociceptive-response curve vs. time. The ratio of antinociceptive area/brain-level area was called the Effect. Then the Effect in mice pretreated with 2.5 g/kg ethanol was divided by the Effect in mice pretreated with water.

| | Naive, s.c. (figure 8) | Naive, p.o. (figure 12) | Tolerant, p.o. (figure 22) |
|---|---|---|---|
| $\frac{\text{Effect}_{ETOH}}{\text{Effect}_{H_2O}}$ = | 2.7 | 2.5 | 2.7 |

Thus, when corrected for differences in brain level, ethanol increased the antinociceptive effect of methadone to a similar extent in both naive and tolerant mice.

The antinociceptive effect of ethanol and methadone combinations is greater than would be expected from simple addition of the observed antinociceptive effects of each drug given alone. For example, the $ED_{50}$ of methadone at 15 minutes is 2.0 mg/kg (figure 5). Addition of the observed effect of 4.0 g/kg ethanol when administered alone (13% MPE, table 2) would result in a decrease in the $ED_{50}$ of methadone to 1.4 mg/kg. However, the $ED_{50}$ of methadone in mice pretreated with 4.0 g/kg ethanol is 0.2 mg/kg. Similar observations can be made based on the observed results in the other dose-response curves as well as the various time-course experiments. Since the combination of ethanol and methadone produced an antinociceptive effect greater than the sum of the effects of ethanol and methadone administered individually, it is appropriate to classify the interaction of these two agents as one of potentiation. This potentiation could be the result of a number of factors.

Just as there are doses of methadone that would produce a degree of antinociception below the sensitivity of the tail-flick, there are doses of ethanol which would also produce a degree of antinociception not detectable with the tail-flick. In view of the activity of doses

of ethanol as low as 1.0 g/kg in some antinociceptive tests such as electrical stimulation of the tail (22), it is possible that 4.0 or even 2.5 g/kg ethanol exerts an antinociceptive effect which is below the sensitivity of the tail-flick, but when combined with methadone, becomes evident as a shift of the methadone dose-response curve to the left. It is impossible to verify the existence of, let alone measure, this subthreshold effect in the tail-flick by its very definition. However, it is possible to estimate its maximum magnitude in order to see if the potentiation of ethanol and methadone antinociception could be due to addition of a subthreshold effect of ethanol to the expected antinociceptive response of methadone.

If there is a subthreshold effect then it is implied that zero activity in the tail-flick test is above zero antinociception. The question then becomes; how far are they separated? If the observed effect of 4.0 g/kg ethanol (13% MPE) is just at, or even slightly above, the tail-flick threshold, then it is possible to answer this question by determination of the amount of methadone required to equal this response.

Since undosed animals would not display any methadone antinociception, that is 0 ng/g = zero antinociception, and from figure 7, 13% MPE would be produced in control animals by a dose of methadone which yielded a brain concentration of methadone of 130 ng/g, the maximum magnitude of the difference in brain-methadone concentration between zero antinociception and the tail-flick threshold is approximately 130 ng/g. Thus, if the decrease in the $EC_{50}$ in ethanol-treated mice was due to a subthreshold effect of ethanol the maximum it could be

decreased by is 130 ng/g. Comparison of the control $EC_{50}$ after subtraction of this maximum possible subthreshold effect with the $EC_{50}$ of methadone in mice pretreated with 4.0 g/kg ethanol reveals that there is still a significant difference between the two groups. This indicates that ethanol has an effect greater than could be produced by simple addition of a possible subthreshold effect of ethanol to the antinociceptive properties of methadone.

The findings that ethanol treated animals display a greater antinociceptive response than controls at the same or even lower whole brain concentrations of methadone, and that this increased response is not due to addition of subthreshold antinociceptive effects of ethanol suggests that the observed potentiation is the result of an ethanol-mediated increase in the apparent CNS sensitivity to methadone. This apparent increase in sensitivity may be due to a number of factors.

Ethanol may increase the effective concentration of methadone at the opiate receptor without necessarily altering the antinociceptive response produced by methadone's interaction with the receptor. Since the distribution of stereospecific opiate binding is not uniform throughout the CNS (85) and it has been shown that the distribution of methadone is not uniform within the brain (122), it is possible that ethanol alters the distribution of methadone within the brain and increases the amount of methadone at these receptors without altering whole brain levels. As opiate binding is predominantly associated with the membrane fraction of brain (120) and ethanol has been shown to increase the fluidity of synaptosomal membranes from the brain (29), it is possible ethanol may enhance the availability of the opiate receptor

for interaction with methadone.

Since the $pK_a$ of all narcotics is above 7.4, the active form of narcotics is thought to be protonated (25). Recent quantum chemical studies of methadone confirm earlier findings that the lowest energy form of protonated methadone is a folded configuration with a hydrogen bond-like interaction of the carbonyl oxygen with the protonated amine to form a pseudopiperidine ring. However, this most stable configuration does not overlap very well with the structure of morphine. A more morphine-like structure, and one which would presumably have a higher affinity for the opiate receptor is an extended chain configuration (95). Since ethanol possesses both good hydrogen bonding capabilities and lipid solubility, it is possible ethanol could favor the formation of this more active conformation of methadone at the receptor and thereby increase the activity of methadone.

Increased levels of calcium have been shown to decrease stereospecific binding of opiates in vitro (121). In agreement with this finding, intraventricular calcium decreases and intraventricular EGTA increases the potency of morphine (63). The demonstration that ethanol produces a rapid and dose-related decrease in brain concentrations of calcium (131, 132) suggests that ethanol may potentiate methadone antinociception by depletion of regional brain calcium.

Finally, as recently reviewed by Takemori (156) increases in the turn-over or concentration of various neurotransmitters such as serotonin, acetylcholine and dopamine have been shown to increase opiate antinociception. Although the results of different studies of the effects of ethanol on these neurotransmitters are far from uniform (78),

owing in part to different doses of ethanol, different species and different methods of analysis, ethanol has been shown to produce increased whole brain levels of all three neurotransmitters in female ICR mice (49). Thus, it is possible that the ethanol potentiation of methadone antinociception observed in the present study is mediated by alterations in one or all of these neurotransmitters.

Although none of the above mentioned mechanisms for an ethanol-mediated increase in the CNS sensitivity to methadone antinociception have been specifically investigated in the present study, the data presented are consistent with this hypothesis. The fact that ethanol produces a parallel shift in the dose-response curves to methadone suggests that methadone is exerting its antinociceptive effect through the same type of receptors in both control and ethanol-treated mice. In addition, as shown in figure 17, increasing doses of naloxone produced equal antagonism of both methadone, and ethanol-methadone antinociception which provides a preliminary indication that ethanol did not alter the naloxone-receptor interaction which in turn implies that ethanol did not alter the configuration of the receptor. However, to validate this it would be necessary to determine the apparent $pA_2$ of naloxone (147) in both ethanol and water-treated mice.

Further support for the hypothesis that the potentiation between ethanol and methadone is due to a change in CNS sensitivity is provided by consideration of some other possible mechanisms of increased antinociception. For example, tetrahydropapaveroline alkaloids or salsolinol have not been demonstrated in vivo after a single dose of ethanol without prior treatment with dopa (161). However, since methadone blocks

dopamine receptors (137) and causes an increase in central dopamine synthesis (19), the formation of these alkaloids may be favored when ethanol and methadone are coadministered. Evidence of antinociceptive activity of both 3-carboxysalsolinol (102) and a derivative of tetrahydropapaveroline (136) supports the possibility that some of the increased antinociception of ethanol and methadone combinations is due to the formation of these alkaloids. However, since the observed potentiation is dependent upon the dose of ethanol and the brain level of acetaldehyde would be the same at different brain concentrations of ethanol (155), these condensation products are probably not involved in the potentiation.

Since high brain concentrations of ethanol increase tail-flick latency, it is possible that the increase in antinociception produced by combinations of ethanol and methadone is due to a methadone-mediated increase in ethanol levels. However, as shown in tables 2 and 10, for subcutaneously administered methadone and in figure 14 for orally administered methadone, methadone does not significantly increase whole brain concentrations of ethanol under conditions where ethanol and methadone produce greater antinociception than methadone alone.

Increased production of active metabolites of d-methadone under the influence of ethanol could also explain the increased activity of ethanol and methadone combinations, but as shown in figure 16, ethanol also potentiates l-methadone, which has no known metabolites with antinociceptive activity (152).

Thus, when all the data are considered, the most reasonable explanation of the ethanol potentiation of methadone antinociception is an

ethanol-mediated change in the CNS sensitivity to methadone's antinociceptive properties.

B) Effect of Ethanol on Brain Concentration of Methadone and Effect of Methadone on Brain Concentration of Ethanol

The present study has investigated the absorption and distribution of methadone in mice and the ability of ethanol to alter these processes, especially as they are reflected in alterations of brain concentrations of methadone. It was found that the brain to serum ratio of methadone in control mice at both a constant time after subcutaneous administration of various doses of methadone (tables 3 and 6) as well as at various times after a single dose of methadone (table 4) was very close to unity. These data are in agreement with the results of a recent study of methadone distribution in mice (141) and contrast with the generally higher brain to serum ratio of methadone found in rats (94, 108).

Pretreatment of mice with 2.5 g/kg ethanol produced brain concentrations of methadone consistently lower than control mice when examined 15 minutes after subcutaneous administration of methadone (table 3). Ethanol pretreatment did not alter the brain/serum ratio of methadone since the lesser brain concentrations of methadone were paralleled by lesser serum concentrations of methadone. When the concentrations of methadone in liver, lung, brain and serum were examined at various times after a subcutaneous dose of methadone (table 4) it was again found that ethanol pretreatment decreased brain and serum methadone at every time point studied. In addition, as shown in table 5, ethanol

pretreatment also produced significant increases in the liver/serum and lung/serum ratios of methadone concentration. The decline in brain and serum methadone can not be explained by decreased absorption since it was found that the absorption of methadone from the subcutaneous injection site was both rapid and unaffected by ethanol pretreatment.

The magnitude of the decrease in brain concentration of methadone was the same after pretreatment with either 2.5 g/kg or 4.0 g/kg ethanol. This raised the possibility that the decreased brain level was caused by acetaldehyde. McCloy et al. (96) have shown, for example, that intra-arterial injection of acetaldehyde dilates vascular beds in the hepatic artery, while constricting those in the carotid and femoral arteries. In addition, intravenous ethanol has been shown to increase heart rate, systemic arterial pressure and myocardial contractile force in dogs (110). While these effects may be responsible for increases in peripheral tissue levels of methadone which could contribute to decreases in brain and serum concentrations of methadone, the observed redistribution of methadone may not be the only factor involved.

As shown in figures 9 and 10 and table 6, when ethanol was administered after, or even simultaneously with the subcutaneous administration of methadone, the brain concentrations of methadone in both ethanol-treated and control mice were generally equal. This study has shown that ethanol is rapidly absorbed after oral administration to mice deproved of food as evidenced by a brain concentration of ethanol of 159 mg/100 g 15 minutes post-administration. Since ethanol metabolism would immediately produce acetaldehyde, if ethanol-mediated redistribution of methadone was the underlying cause of alterations in brain and

serum methadone, these alterations would be expected to be similar whether ethanol was administered simultaneously with, or administered prior to, methadone.

While the response to acetaldehyde is immediate, other effects of ethanol are more delayed. For example, ethanol produces a marked diuretic response due to its ability to suppress antidiuretic hormone release (162). However, the diuresis is short lived and is followed by a period of decreased urine formation (106). Thus, pretreatment with ethanol may produce a diuretic response at the same time methadone is being rapidly absorbed and serum concentrations of methadone are high and thereby increase the initial excretion of methadone. The effect of ethanol on excretion may be magnified since methadone decreases urine formation (72) and control mice may be producing a lower than normal urine volume at the same time ethanol-treated mice are producing a greater than normal urine flow. When ethanol and methadone are administered simultaneously, the diuretic effect may only develop after serum concentrations of methadone are much lower and therefore the increased urine volume is less effective in producing noticeable changes in brain and serum concentrations of methadone. The fact that the effects of 2.5 and 4.0 g/kg ethanol are similar may indicate that 2.5 g/kg maximally inhibits antidiuretic hormone release (34). Further study of the effects of ethanol and methadone combinations on urine formation and the excretion of methadone are needed to clarify the role of excretion in the observed decrease of brain concentrations of methadone.

When ethanol and methadone are administered orally, initial brain concentrations of methadone are approximately 40% less than in control

mice (figure 12). This decrease is probably due to the same effects noted following subcutaneous administration as well as an additional effect of ethanol to initially decrease absorption of methadone from the gastrointestinal tract. As shown in table 8, both 2.4 and 4.0 g/kg ethanol delay the loss of methadone from the stomach to a similar extent. Studies of the oral absorption of methadone in rats with or without a pyloric ligation show that although some methadone is absorbed from the stomach, the greater portion is absorbed from the small intestine which makes gastric emptying the rate-limiting step in the oral absorption of methadone (170). Since ethanol is known to inhibit gastric emptying when administered by either the oral (8) or intravenous (59) route but does not alter absorption of methadone once methadone is in the duodenum (170), the ethanol-mediated initial decrease in gastrointestinal absorption of methadone is evidently produced by delayed entry of methadone into the small intestine. Thus ethanol, by decreasing gastric emptying prevents the rapid absorption of methadone seen in controls and produces a more gradual and sustained absorption of methadone which results in brain concentrations of methadone initially lower and finally greater than controls. As can be seen in figure 12, both 4.0 and 2.5 g/kg ethanol produced the same effects on brain concentration of methadone. This is probably due to the use of the same concentration of ethanol to dose both groups and as mentioned above, both doses produce similar effects on the distribution and excretion of methadone once it is absorbed.

As shown in figure 15, the half-life of methadone in the brain after subcutaneous administration was not altered by pretreatment with

2.5 g/kg ethanol. When considered in conjunction with the lack of effect of ethanol treatment on the relative amounts of methadone, EDDP, and EMDP excreted in bile and urine, these observations suggest that this dose of ethanol does not significantly alter *in vivo* metabolism of methadone. Supplementary information on the effect of ethanol on methadone metabolism *in vitro* is lacking although Cohen and Mannering (35) found ethanol did not alter N-demethylation of methadone at concentrations which decreased aniline *para*-hydroxylation. However, since ethanol is a more potent inhibitor of the metabolism of type II compounds (aniline) than of type I compounds (methadone) and they did not investigate higher concentrations of ethanol, their investigation does not necessarily support the concept that ethanol does not alter methadone metabolism *in vivo*.

It is possible that the lack of effect of ethanol on the half-life of methadone in brain reflects an ethanol-mediated inhibition of methadone metabolism which is masked in the whole animal by other effects of ethanol. For example, the observed increased concentration of methadone in the liver of ethanol-treated mice may compensate for an ethanol-mediated inhibition of methadone metabolism. In mice maintained on an oral dose of methadone of 20 mg/kg/day, the oral administration of ethanol (2.5 g/kg) prior to the last dose produced an increase in the half-life of methadone in brain compared to controls (figure 23). However, this alteration in half-life may not be due to an ethanol inhibition of methadone metabolism but due to an initial delay in methadone absorption as was shown in naive mice deprived of food. To circumvent this possibility, it would be useful to study the effects of ethanol in mice

made tolerant to methadone by subcutaneous administration. In addition, further work on the in vitro metabolism of methadone in both naive and methadone-tolerant mice is needed to clarify the effects of ethanol on methadone metabolism.

In contrast to the significant effects of ethanol on brain concentrations of methadone, especially when both drugs are administered orally, methadone generally produced only slight alterations in brain concentrations of ethanol. In fact, as shown in figure 14, the only significant change produced by a 30 minute oral pretreatment with methadone was a lower brain concentration of ethanol 15 minutes post ethanol. There is also some indication of a delay in the time of peak concentrations of ethanol, however, the rate of decline in brain concentrations of ethanol is little affected by methadone. As shown in tables 2 and 10, subcutaneous administration of methadone also has little effect on the brain concentrations of ethanol. The alterations observed are probably a reflection of the anticholinergic properties of methadone (171) which would delay gastric emptying in agreement with the effects of other drugs with anticholinergic properties on ethanol absorption (41, 54, 60). Since ethanol is well absorbed from the stomach even when the stomach is ligated at the pylorus (79), it is to be expected that alterations in the rate of gastric emptying have a greater effect on methadone absorption than on ethanol absorption.

C) Toxicity of Ethanol and Methadone Combinations

In view of the common CNS depressant properties of ethanol and methadone, it would not be surprising to find an increase in the toxicity of methadone when combined with ethanol. However, as shown in figure 24, pretreatment with ethanol did not significantly alter the observed $LD_{50}$ of orally administered methadone until an ethanol dose of 4.0 g/kg was employed. It would seem likely that ethanol would alter the absorption of lethal doses of methadone in a fashion similar to pharmacological doses, and therefore insight into the effects of ethanol on the $LD_{50}$ of methadone could be gained by referring to the effects of ethanol pretreatment upon brain concentrations of methadone shown in figure 12. Control animals rapidly achieved high brain concentrations of methadone, while ethanol pretreatment suppressed this initial rapid absorption and produced a more gradual increase in brain concentrations of methadone. Thus, even at the same dose of methadone, the maximum brain concentration of methadone achieved by ethanol-pretreated mice is much lower than the maximum achieved by controls. Due to these ethanol-mediated alterations in brain concentration of methadone, the increased toxicity of ethanol and methadone combinations is seen not as a shift in the $LD_{50}$, but as a significantly lower concentration of methadone in the brain at death as shown in table 16.

The ability of ethanol to alter brain concentration of methadone may also be partly responsible for the observed increased latency of death in ethanol-pretreated mice. Since ethanol delays the increase in brain concentration of methadone, ethanol-pretreated mice require a

longer time to achieve a lethal concentration of methadone even though this concentration is less than in control mice. Since the effects of 4.0 and 2.5 g/kg ethanol on the time course of brain concentration of methadone are similar, but the latency of death in mice pretreated with 4.0 g/kg is significantly greater than when mice are pretreated with 2.5 g/kg, ethanol may increase the latency of death by other mechanisms in addition to initially decreasing brain concentrations of methadone. For example, in addition to CNS depression, methadone displays some CNS stimulant properties in mice as evidenced by its ability to increase locomotor activity (figure 19). During the $LD_{50}$ experiment, it was observed that ethanol-pretreated mice did not seem to exhibit as much locomotor activity as control mice. Thus, ethanol may have increased the latency of death by initially protecting the mice from the stimulant properties of methadone.

Since mice pretreated with 2.5 g/kg ethanol did survive slightly longer than controls, the decreased brain concentration of methadone at death may not necessarily reflect an initial delay in methadone delivery to the brain, but could be due to the longer time the animal was alive and metabolizing methadone. To evaluate this possibility methadone was administered subcutaneously. Under these conditions both ethanol-treated and control mice died at approximately the same time. As shown in table 16, the brain concentrations of methadone in the mice that died in the ethanol pretreated group was still significantly lower than in the controls.

These experiments indicate that it took a lower brain concentration of methadone to be lethal in mice pretreated with ethanol, but

since ethanol also decreased the percent of the dose which reached the brain, the $LD_{50}$ was not altered until higher doses of ethanol were employed. Thus, although 2.5 g/kg ethanol increased the antinociceptive response to such an extent that the dose-response curve was shifted to the left in spite of a lower brain concentration of methadone, this same dose of ethanol did not increase the toxic effects of methadone enough to overcome the decrease in brain concentration.

It was observed that combination of a 2.5 g/kg dose of ethanol with the daily dose of methadone in mice maintained on 100 mg/kg/day produced as many deaths as were produced by doubling the daily dose of methadone. Further investigation of the $LD_{50}$ of ethanol in methadone tolerant mice (table 17) revealed that, as might be expected, the higher the maintenance dose of methadone, the lower the $LD_{50}$ of ethanol. These findings may parallel the methadone maintenance patient who combines ethanol and his daily methadone dose and succumbs to their combined effect when the daily dose of methadone alone would have been tolerated.

## SUMMARY

Ethanol causes a significant potentiation of the antinociceptive properties of methadone in both naive and methadone-tolerant mice as measured in the tail-flick procedure. This increased antinociception is not due to an ethanol-mediated increase in whole brain concentration of methadone. Ethanol pretreatment was found to cause a significant decline in whole brain concentrations of methadone. It is hypothesized that the increased antinociceptive response to combinations of ethanol and methadone is the result of an ethanol-mediated increase in central nervous system sensitivity to methadone.

Ethanol treatment does not alter the half-life of methadone in the brain, nor does it alter the relative amounts of methadone and its major nonconjugated metabolites excreted in the bile or urine of naive mice.

Ethanol pretreated mice die at a lower brain concentration of methadone than mice that die as a result of methadone alone. However, since ethanol also alters the percent of the methadone dose which reaches the brain, the $LD_{50}$ of methadone is not altered by pretreatment with an ethanol dose of 2.5 g/kg. In mice maintained on high daily doses of methadone, there is a decline in the $LD_{50}$ of ethanol.

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# APPENDIX I

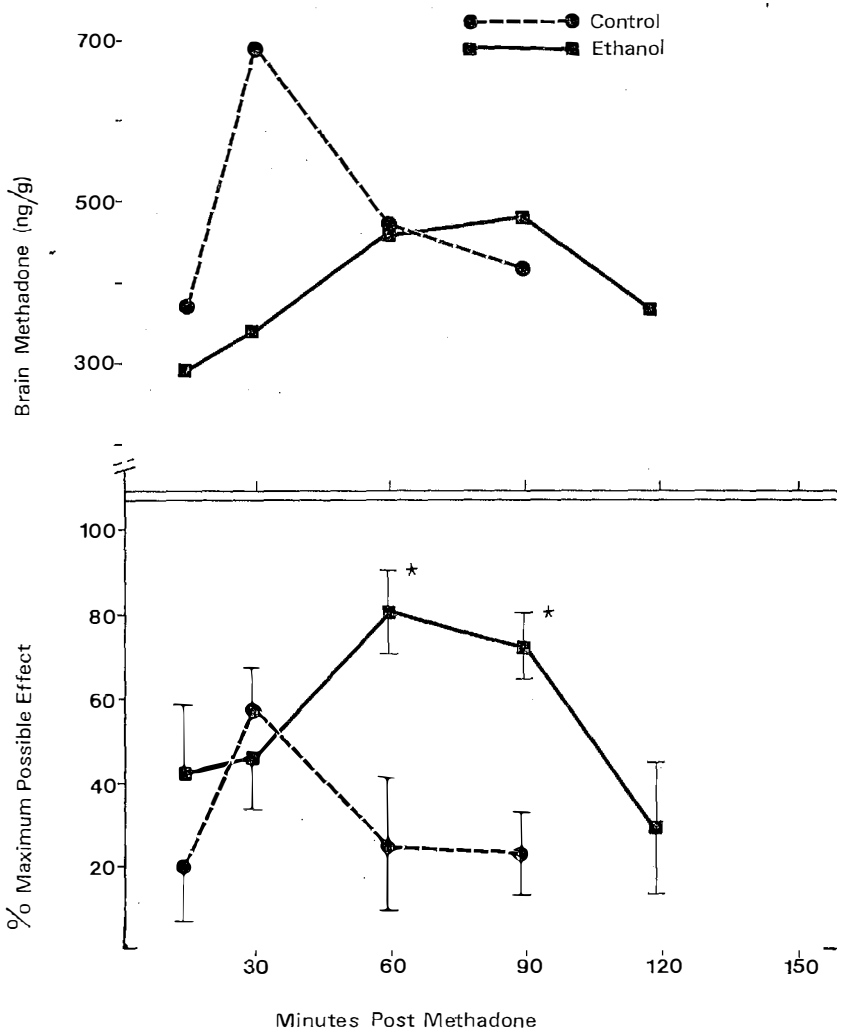
## Determination of Methadone Concentration In Brain

In addition to the $^{3}H$ and GLC determinations of methadone in brain presented in figures 2 & 3 respectively, a combined GLC/$^{3}H$ assay was employed in order to check the accuracy of brain-methadone determinations based on the $^{3}H$-label. This procedure was identical to the GLC assay except promethazine was used as an internal standard instead of SKF-525A and the initial solvent extract was split. A 5 ml aliquot was evaporated and counted to determine the apparent amount of $^{3}H$-methadone. This was corrected for $^{3}H$-methadone metabolites by using the appropriate factor derived from previous experiments. The remaining solvent was back extracted with $H_2SO_4$ and processed as in the GLC procedure. In this way, brain levels based on GLC and on analysis of the $^{3}H$-label could be determined in the same sample. Since promethazine decomposes to phenothiazine, it was necessary to purify the stock internal standard solution each day before use. This was accomplished by TLC separation (154). The promethazine was extracted from the silica gel with a small amount of ethanol and then diluted to the appropriate concentration with water. The GLC results from two brain samples were confirmed by GC/MS as described in methods. The average ratio of GC-MS/GC levels was 0.93.

Using this $^{3}H$/GLC procedure, the brain concentrations of methadone were determined in mice administered either water or 2.5 g/kg ethanol

FIGURE 27

EFFECT OF ETHANOL ADMINISTERED PRIOR TO METHADONE




Ethanol (2.5 g/kg, p.o.) or water was administered 30 minutes prior to methadone (8 mg/kg, p.o.). Each point represents the mean ± SEM of six mice. Brains were pooled in groups of three to determine methadone by GLC.
* significantly different from control at $P < .05$

thirty minutes prior to oral administration of methadone (8 mg/kg). This dosing protocol was identical to that used in figure 12. In this duplicate experiment the GLC brain methadone levels (figure 27) showed that water-pretreated mice exhibited a rapid absorption of methadone peaking at thirty minutes while ethanol-pretreated mice showed a delayed absorption with brain methadone levels initially lower and finally greater than control. These results confirm the time course of brain methadone levels based on $^{3}$H-analysis found in the previous set of animals (figure 12). In addition, the $^{3}$H-methadone concentrations from the combined $^{3}$H/GLC procedure indicated exactly the same trend. It was observed, however, that the absolute magnitude of the $^{3}$H-methadone concentrations tended to be lower than the concentrations calculated on the basis of the GLC assay.

To investigate the magnitude and mechanism of this difference, in addition to comparison of brain methadone levels from the two assays, the specific activity of the methadone recovered from the brains was also determined. The calculation of this final specific activity was accomplished by determination of the number of nanograms of methadone in identical aliquots of the final extract by both GLC and scintillation counting. In both cases, the samples were compared to known aliquots of $^{3}$H-methadone from the injection solution on the basis of absolute methadone peak height or DPM. The remaining extract was analyzed by TLC to obtain the correction factor for total DPM to methadone DPM. Since the GLC determination of absolute nanograms of methadone relies on the absolute peak height of methadone and it was found that a small amount of promethazine decomposes to phenothiazine during the process of

extraction, and further, that phenothiazine elutes very close to methadone, the internal standard for this and all subsequent GLC analyses was changed to SKF-525A.

Although this made the determination of specific activity more accurate, it was found that SKF-525A is extracted approximately half as efficiently from mouse brains homogenates as it is from human brain homogenates although methadone is extracted equally well from both. The same type of problem has been noted by at least one other worker (23) who found the extraction of methadone to be equally efficient in all tissues studied but also noted that SKF-525A extracted with a different efficiency in each type of tissue studied (brain, liver, lung, spleen, kidney). Due to this difference in the extraction of SKF-525A, at least one standard prepared in mouse-brain homogenates was coextracted with each group of samples and human-brain standards. The standard curve established with human-brain standards was corrected for the difference in extraction of the internal standard by the mouse-brain standards. This difference was very reproduceable from day to day. The average ratio of peak height ratios (methadone/SKF-525A) between mouse and human brain standards was 2.06 $\pm$ .07 based on five standards over a three week period.

Table 19 presents the alterations in specific activity as well as the $^{3}H$/GLC results from the analysis of brains from mice pretreated with ethanol or water thirty minutes prior to the oral administration of methadone (8 mg/kg). These results confirm the reality of the difference between $^{3}H$ and GLC methadone concentrations and show that the observed differences in $^{3}H$ and GLC concentrations are caused by a

TABLE 19

TIME COURSE OF ALTERATIONS IN SPECIFIC ACTIVITY

| Minutes post methadone (Pretreatment) | Specific activity | $^{3}H$/GLC |
|---|---|---|
| 30, (water) | 0.80 ± .05 | 0.78 ± .09 |
| 30, (ethanol) | 0.83 ± .08 | 0.81 ± .05 |
| 90, (water) | 0.68 ± .10 | 0.71 ± .06 |
| 90, (ethanol) | 0.74 ± .06 | 0.75 ± .07 |

There were three samples /dose with two mouse brains/sample. Mice were pretreated with either water or ethanol(2.5g/kg, p.o.) 30 minutes prior to recieving $^{3}H$-l-methadone diluted with unlabelled d,l methadone ( 8 mg/kg, p.o.).

decrease in the specific activity of methadone and were not due to improper application or calibration of either analytical method. In addition, these results demonstrate that the magnitude of the decrease thirty minutes post administration was almost the same as at ninety minutes and was not altered by pretreatment with ethanol.

Since all the radiolabled methadone was l which had been diluted with unlabled d,l-methadone, one of the reasons for the decreased specific activity could be stereoselective metabolism of methadone or a more rapid metabolism of l-methadone than d-methadone. Although Sung and Way (153) found that each enantiomer of methadone had the same brain half-life, other workers (15, 107, 157) showed that the metabolism of d-methadone is faster than l, which would, if anything, tend to increase the specific activity. These studies were all done with each enantiomer separately and, as shown by Taylor (157), when the racemic mixture was studied, the metabolism of l-methadone was favored over that of d-methadone. Beckett (15) suggested that this could be explained by l-methadone possessing a higher affinity for the metabolizing enzyme but a slower matabolic rate than d-methadone. If this is true, l-methadone would inhibit the metabolism of d-methadone and this would tend to decrease the specific activity of $^{3}$H-l-methadone diluted with d,l-methadone.

Table 20 shows that when $^{3}$H-l-methadone diluted with d,l-methadone was administered subcutaneously rather than orally, there was no alteration in the specific activity, and there was a very close agreement between $^{3}$H and GLC methadone concentrations. This tends to discount the importance of stereoselective metabolism as

TABLE 20

CHANGES IN SPECIFIC ACTIVITY OF $^{3}$H-METHADONE

| | Ratio of final/initial specific activity | $^{3}$H brain level GLC brain level |
|---|---|---|
| d,l Methadone | | |
| s.c. | 1.02 ± .05 | 0.97 ± .05 |
| p.o. | 0.71 ± .08 | 0.73 ± .05 |
| l Methadone | | |
| s.c. | 1.03 ± .07 | 1.06 ± .03 |
| p.o. | 0.78 ± .05 | 0.70 ± .07 |

Specific activity and brain methadone levels were determined 90 minutes after methadone was administered ( 8 mg/kg p.o. or 2 mg/kg s.c.).

the cause of the decreased specific activity seen after oral administration. In addition, as also shown in table 26, when $^{3}$H-l-methadone was diluted with unlabeled l-methadone the specific activity still decreased after oral but not after subcutaneous administration, further indicating a mechanism other than stereoselective metabolism. The data that indicate no alteration in the specific activity of $^{3}$H-l- methadone diluted with unlabeled d,l-methadone are in agreement with the results of Alvares and Kappas (3) and Sullivan et al. who found no difference in the in vitro rate of metabolism of methadone isomers in rat liver microsomes (151).

It is possible that there is an irreversable stereoselective binding of methadone to the "narcotic receptor" or other brain constituents which favors l-methadone and thus decreases the specific activity of recovered methadone. The decreased specific activity after oral administration of $^{3}$H-l-methadone diluted with l- methadone and the lack of alteration of specific activity of $^{3}$H-l-methadone diluted with d,l-methadone after subcutaneous administration discounts stereoselective binding as a reason for decrease in specific activity.

The most likely explanation for the observed alterations in specific activity is exchange of the $^{3}$H-label with water. This exchange could conceivably occur during the extraction of the samples but since the specific activity of the standards which were processed in parallel with the samples was unaltered, this does not seem likely. To determine if the exchange was taking place during storage or homogenization of the brains, aliquots of an injection solution were injected into

several sites of four blank mouse brains such that each brain received 10 µl of solution. The brains were then frozen and two were analyzed after two days of storage. the remaining two were analyzed after ten days of storage. In both cases the specific activity of methadone was found to be unaltered. In addition, since all brain samples were stored, homogenized and extracted in the same manner but alterations of specific activity were only observed in mice dosed orally, these procedures cannot be the source of the exchange. In view of the alteration in specific activity following oral but not subcutaneous administration, the exchange with water is probably occuring in the gastrointestinal tract.

Evaluation of the exchange of $^{3}H$-methadone was monitored by measuring the amount of $^{3}H$ which was extracted by EDC/isopropanol before and after incubation in water. Incubation of $^{3}H$-methadone in water at 35$^{o}$C resulted in a 5% decrease in extractable radioactivity after 30 minutes but no further decrease in the next 24 hours. Incubation at pH 7.4 and 35$^{o}$C did not result in any measurable decline in 24 hours. Information from the manufacturer of the $^{3}H$-methadone used (113) indicated that approximately 5% of the $^{3}H$-lable is not in the 1 position. Since the $^{3}H$-methadone is produced by reaction of 1-4-dimethylamine-2,2-diphenylvaleronitrile with ethyl magnesium bromide (2-$^{3}H$), it would seem likely that this 5% is in the 2 position next to the carbonyl group. This position would be very labile and in fact exchange at this position of methadone has been observed (66). This type of exchange could explain the observed in vitro decrease in extractable $^{3}H$. However, in addition to instability in vitro, there must be an

additional exchange in vivo since the specific activity of recovered methadone is decreased by approximately 30%. Since the decrease of specific activity is not affected by pretreatment with ethanol (table 14) which causes a delay in the loss of methadone from the stomach (table 8) this additional exchange may occur in the small intestine.

The data presented here suggest that following oral administration of $^{3}H$-methadone there is an exchange of the $^{3}H$-label which results in an approximate 30% decrease in specific activity. The same decrease in specific activity is not observed following subcutaneous administration. The decrease in specific activity is not affected by pretreatment with ethanol and furthermore, the decrease at 30 minutes post methadone is almost the same as at 90 minutes. In experiments where both $^{3}H$ and GLC methods were used to determine methadone concentration in the brain after oral administration it was found that each assay showed the same relative magnitude between ethanol and water pretreated animals and a similar time course of brain methadone levels. However, the absolute magnitude of the $^{3}H$-levels was lower due to the change in specific activity. Although it would have been valid to correct the $^{3}H$-methadone concentrations for alterations in specific activity and report these results as ng of methadone/g of brain, this was not done. Results based on $^{3}H$-analysis after oral administration of methadone are reported as DPM/g. The results after subcutaneous administration are reported as ng methadone/g since no alteration of specific activity occured with this route of administration.

# APPENDIX II

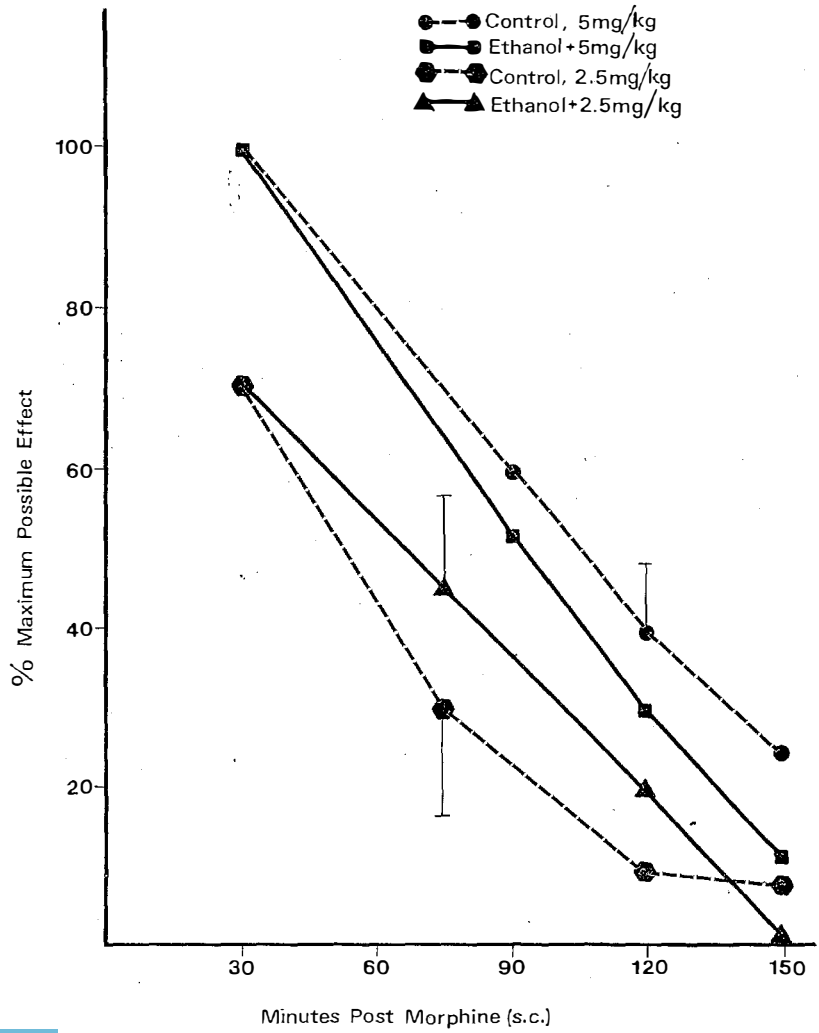
The results of administration of ethanol 30 minutes prior to the subcutaneous administration of morphine, propoxyphene or meperidine are presented in figures 28, 29 and 30 respectively. This dosing protocol is identical to that used for methadone in figure 8. Under these conditions, the brain concentration of methadone was decreased relative to control concentrations.

If the factors which produced this decline in brain concentrations of methadone also decreased brain concentrations of these other narcotics to a similar degree, then the antinociceptive properties of these agents would also seem to be increased by ethanol pretreatment. If the brain concentration of these agents was decreased to the same extent as methadone, these other narcotics would seem to be potentiated to a lesser degree than methadone in that ethanol increased methadone antinociception to such an extent that even in the face of decreased brain concentrations of methadone, the ethanol and methadone antinociceptive response was greater than control. Ethanol and these other narcotics generally produced antinociceptive responses equal to control responses.

On the other hand, if ethanol pretreatment does not alter the brain concentration of these agents, then it would seem that ethanol is unable to increase the antinociceptive properties of these narcotics. Further investigation of the combined effects of ethanol and these narcotics is needed to differentiate between these possibilities.

FIGURE 28

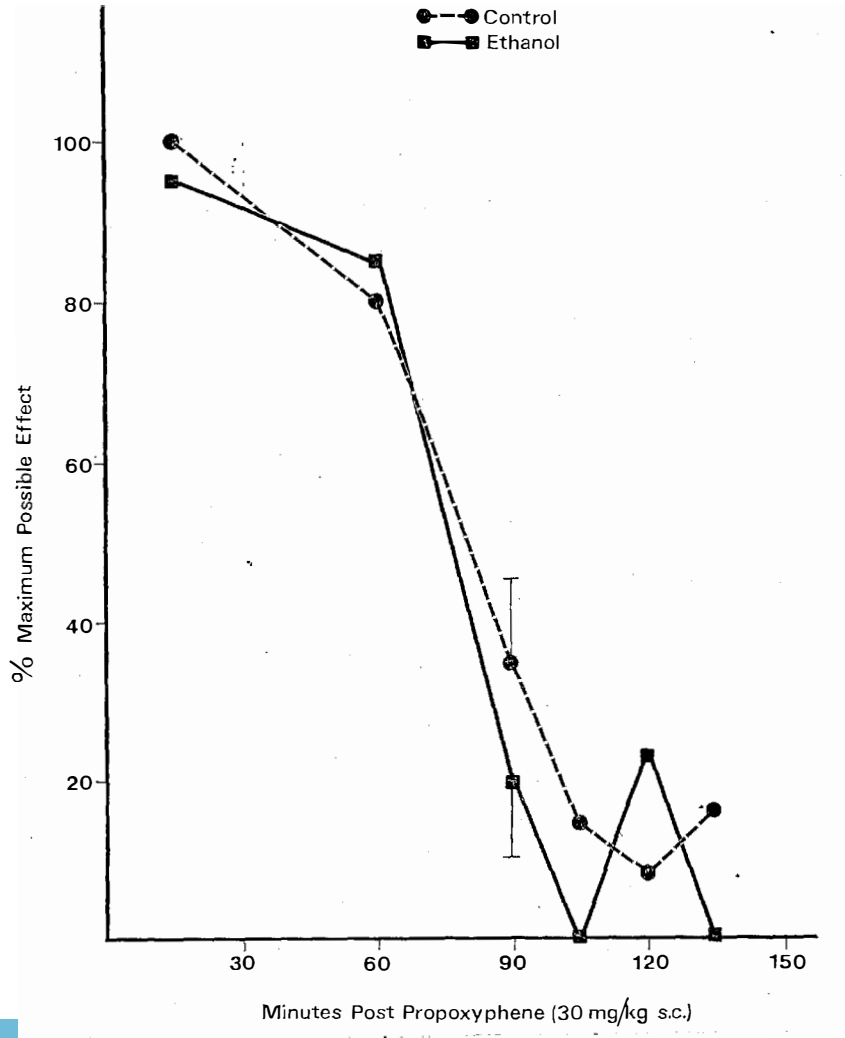
THE EFFECT OF ETHANOL ON THE TIME COURSE OF THE TAIL-FLICK ACTIVITY OF MORPHINE




Ethanol (2.5 g/kg, p.o.) or water was administered 30 minutes prior to morphine. There were six mice/dose and each mouse was tested at the indicated times. The SEM shown are representative of the SEM at the other time points.

FIGURE 29

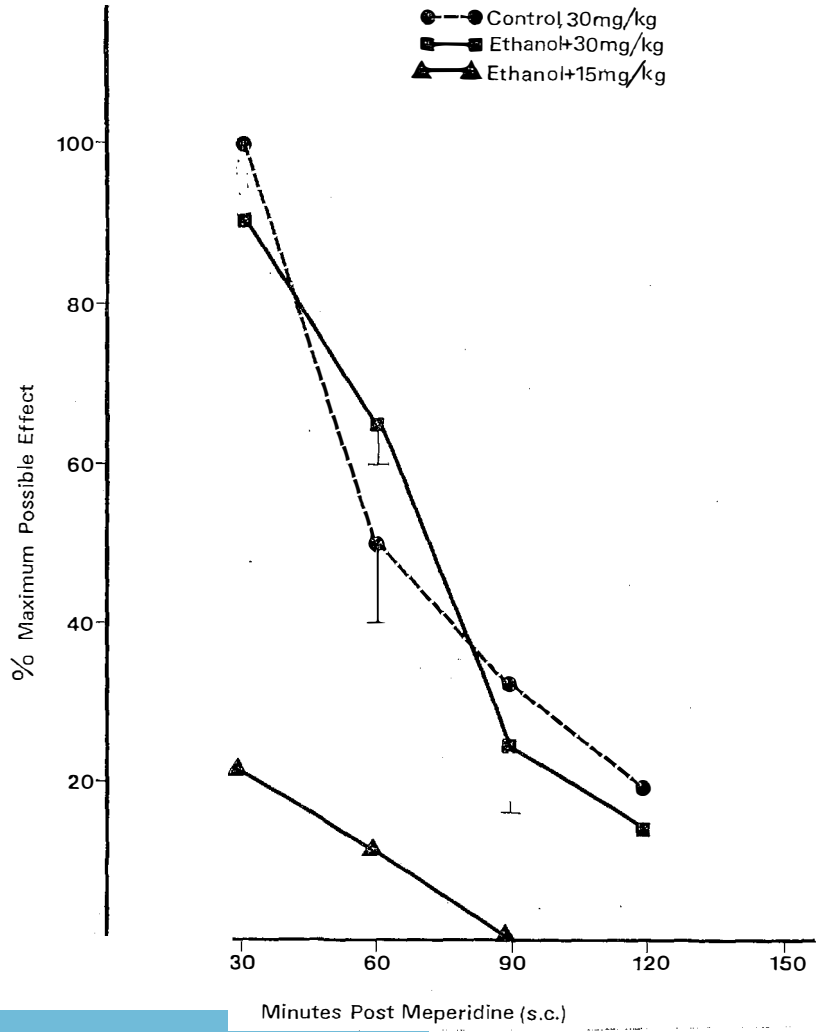
THE EFFECT OF ETHANOL ON THE TIME COURSE OF THE TAIL-FLICK ACTIVITY OF PROPOXYPHENE




Ethanol (2.5 g/kg, p.o.) or water was administered 30 minutes prior to propoxyphene. There were six mice/group. Mice tested at 60 minutes were retested at 120 minutes. Mice tested at 90 minutes were retested at 135 minutes. The SEM shown are representative of the SEM in the other groups.

FIGURE 30

THE EFFECT OF ETHANOL ON THE TIME COURSE OF THE TAIL-FLICK ACTIVITY OF MEPERIDINE




Ethanol (2.5 g/kg, p.o.) or water was administered 30 minutes prior to meperidine. There were six mice/dose and each mouse was tested at the indicated times.