THE INVOLVEMENT OF CHOLINERGIC MECHANISMS IN MORPHINE DEPENDENCY

A Thesis Presented to The University
of Manitoba in Partial Fulfillment
of the Requirements for the Degree of
Doctor of Philosophy

by

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ABSTRACT

Everyone is motivated by the search for pleasure and for the relief of pain. Narcotic use and abuse probably date to the ancient Egyptians. Despite their popularity and lengthy history, a clear understanding of their mechanism of action has not yet been forthcoming. There has, however, been no shortage of theoretical speculation. Prominent amongst these hypotheses is the tenet that cholinergic pathways play an important role in the actions of the opiates. In the present work an attempt has been made to further elucidate the former's participation in the phenomena of morphine dependency and withdrawal. In addition, work presented here was amongst the first studies performed in search of endogenous opiates and the methodology provided the basis for many future investigations.

Choline chloride, a partial cholinergic agonist, had previously been shown to ameliorate the withdrawal syndrome in rats. A more objective, all-or-none quantitation was used here to further delineate this beneficial effect. Antagonist-precipitated jumping in mice, as well as in rats, was significantly reduced in choline pretreated animals. Also, the optimal dose of this partial agonist differed as a function of the rate and extent of development of morphine dependency.

The toxicity of choline, given acutely, as determined by its LD50, displayed the classical sigmoid shape. Successive sub-lethal doses appeared to have cumulative toxicity, with animals exhibiting increased sensitivity during nighttime hours. This agrees with results of other investigators who have shown a diurnal sensitivity for other cholinergic

agents.

In an attempt to monitor changes in cholinergic sensitivity during the development of morphine dependency as well as during the abstinence syndrome, the tremorigenic agents, oxotremorine and harmine, were injected into animals at various times during and after chronic morphine administration. There was a marked variation in tremor response throughout the morphine cycle with oxotremorine (classically cholinergic in nature) whereas no variation occurred with harmine (noncholinergic in action). In addition, a choline-oxotremorine interaction was observed, with modification by atropine, whereas no such relationship was observed between choline, harmine, and propranolol.

Although it is difficult to confidently extrapolate the beneficial effects of choline chloride from rats to man, in spite of a suggestion of such an effect seen in a small unblinded study conducted during the course of this work, it appears to be a worthwhile venture to search for longer-acting partial cholinergic agonists and assess their value in larger-scale human studies.

Towards the end of these studies, interest was mounting in the search for endogenous opiates. Experiments conducted here were based on the hypothesis that such a substance might be a gonadal or adrenal steroid and that they may be mobilized during sustained, mildly noxious stimuli. The effects observed after gonadectomy and/or adrenalectomy are compatible with such a possibility. Furthermore, using similar testing procedures, an anomalous, antinociceptive effect of naloxone was observed at very low doses. This result represents one of the earliest demonstrations of an interaction between an exogenous opiate antagonist and a deliberate experimental provocation of endogenous opioid release.

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I INTRODUCTION

(A) HISTORY AND GENERAL PHARMACOLOGY OF MORPHINE

The search for pleasure and for the relief of pain motivates everyone. From the beginning of recorded history, drugs have played a prominent role in pleasurable recreational activities and in relieving various forms of discomfort, both mental and physical (Sapira, 1975). Ancient Sumeria has often been credited with the origin of opium, referred to by the ideograph "hul-gil" or "joy plants" (Jaffe and Martin, 1975), but its use and abuse probably date to the more ancient Egyptians as evidenced by the decoration of tombs with the poppy (Sapira, 1975).

Morphine is the prototype of a family of chemicals generally referred to as the opiate narcotics. It is one of many alkaloids extracted from the juice of the unripe poppy plant, Papaver somniferum, and is a member of the class phenanthrenes. Morphine is readily absorbed from the gastrointestinal tract but its effects are less after oral administration than after parenteral administration. Only small quantities pass the blood-brain barrier under normal conditions. It is conjugated with glucuronic acid and most of it is excreted in the urine along with small amounts of free morphine (Jaffe and Martin, 1975).

A variety of semisynthetic drugs are made from morphine by simple substitution. Thus, agonists such as codeine and heroin, and antagonists such as nalorphine and naloxone are easily derived from the parent compound.

Morphine exerts its actions on virtually every organ system in man. Centrally, it causes analgesia, mood changes, drowsiness, mental clouding and ultimately, sleep. These effects are dose-dependent so that it is possible to achieve significant analgesia without loss of consciousness. The analgesic effect is selective in that the responsiveness of other

sensory modalities is not diminished. The type of pain is differentially affected by morphine, continuous dull pain being alleviated more easily than sharp intermittent pain. The mechanism of action of analgesia is still unknown but it appears that it, is the reactive component, i.e. the "appreciation" of pain, that is altered and not the pain sensation itself.

The effects of the opiates on the gastrointestinal tract have been recognized for centuries. These drugs cause an increase in the tone of the antral part of the stomach as well as of the first part of the duodenum in addition to a decrease in motility of the former. This results in a delay of passage of gastric contents and constitutes a major component of the constipation observed with morphine administration. There are similar effects on intestinal tone where increases are seen in the small and large intestine, ileocecal valve and anal sphincter. In addition these periods of hypertonicity may be followed by periods of atony. Propulsive contractions are diminished in all areas of the intestine and this further contributes to the constipating actions. Large doses of atropine can antagonize the spasmogenic effects; extrinsic nerve resection and ganglion blocking drugs do not, suggesting that it may be nerve plexuses within the bowel wall which are being affected (ibid). The smooth muscle contraction produced by morphine-like drugs may exacerbate an attack of biliary colic instead of alleviating the pain in this condition. Ureteric tone and contractions are increased as are detrusor tone in the urinary bladder and vescical sphincter, the latter two phenomena causing difficulty in urination.

The most serious consequences of excessive morphine administration are those of respiratory depression. All phases of respiratory activity

are decreased even with therapeutic doses and at least part of this effect is due to a direct action on the brainstem resulting in a diminished response to plasma PCO_2 and to a disruption in the regular rhythmicity of respiration (ibid).

Direct stimulation by morphine of the chemoreceptor trigger zone (CTZ) in the area postrema of the medulla causes nausea and vomiting, evidence of which is more common in ambulatory than recumbent patients, suggesting that there is also a vestibular component to these effects. Morphine and its derivatives also depress the cough reflex at least partly by a direct effect on the cough center in the medulla (ibid).

Pupillary constriction in man persists even after tolerance has developed to other actions. There is considerable species variation in the pupillary response to morphine but these responses do not change with repeated drug administration (ibid).

The cardiovascular system is little affected by therapeutic doses of morphine although orthostatic hypotension and fainting do occur. There is evidence from animal studies that this effect may involve morphine-induced inhibition of central noradrenaline neurotransmission (Gomes et al., 1976).

(B) DEFINITION OF TERMS: THEORIES OF MORPHINE DEPENDENCY

(a) General. The mechanisms of the development of tolerance to and physical dependence on the opiate narcotics are still unknown but there certainly has been no shortage of theoretical speculation. Although the terms tolerance and dependence are often mentioned together, implying that they are similar in definition, they are, in fact, quite different phenomena.

Tolerance, as defined by Seevers and Woods (1953), is "cellular

adaptation to an alien chemical environment characterized by diminished biological response". Tolerance to a drug, therefore, is demonstrated only during repeated administration of that drug (or of a similar drug - "cross tolerance"). Physical dependence, on the other hand, is a state which is inferred from the signs of physiological dysfunction that occur only after discontinuation of drug administration; these signs are reversed upon re-administration of the drug (Schuster and Balster, 1973). Almost all drugs that cause dependence also produce tolerance. It is important, however, to point out that not all drugs which produce tolerance will necessarily cause dependence.

In addition to the foregoing definitions, the two phenomena have also been differentiated by their time course for development. Tolerance was considered to have a shorter time course for development than did physical dependence. Several papers, however, have shown that physical dependence can occur very quickly and that a single low dose of morphine is sufficient to cause measurable dependence (Barthelemy and Jacob, 1972; Kosersky et al., 1974; Frumkin, 1974; Smits, 1975). These results suggested that the classical concept, which holds that a prolonged period of drug administration is necessary for the induction of opiate narcotic dependence, may no longer be tenable.

Several theories have been advanced to explain the phenomena of opiate tolerance and dependence, they are not necessarily mutually exclusive but may merely be dealing with the phenomena in different terms. There are two major approaches, however, that have emerged as contending theories and have received considerable attention. The first of these postulates a continuous and unchanged interaction between the drug and

its receptors, the effects of which are compensated or antagonized by changes in other biochemical pathways or in other neuronal systems. The second theory attributes the drug tolerance to some change in the drug receptors themselves - either a change in their number or a change in their properties - making them less sensitive to the drug. A brief review of the prominent theories now follows:

(b) Theories.

(i) Pharmacological Redundancy. Martin (1970) claimed that redundancy is a universal biological process and that it is required for the continuous functioning of systems that have been exposed to injurious forces. The main features of his theory are (1) the existence of "primary" neural pathways that mediate normal function and of "redundant" pathways which are not activated during normal activity; these pathways run in parallel; (2) that these pathways are differentially sensitive to chemical agents and both are subject to tonic inhibition from effector tissues; (3) that blockade of the "primary" pathway by a chemical agent will cause a decreased negative feedback to the "redundant" pathway which will result in its activation and eventual "hypertrophy" (leading to chronic tolerance); and (4) when the selective blockade is removed, the activity of the "primary" pathway is restored along with the maintained activity of the "hypertrophied redundant" pathway resulting in the abstinence syndrome which is the objective expression of physical dependence.

It should be pointed out however, that mere occupation of receptors by a drug in the "primary" pathway is not sufficient to induce the "hypertrophy" of the "redundant" pathway; the drug must exert anonistic effects. Hence, a "pure" antagonist such as naloxone would not be expected to produce physical dependence. This theory is highly speculative and although

there are many examples of "redundancy" in other systems, such as enzyme shunts and bilateral symmetry of organs, it is very difficult to find data that will unequivocally support or refute Martin's postulates.

(ii) Dual Action. This theory is based on the fact that morphine and its derivatives exert a dual action in man and animals, as originally reported by Tatum et al. (1929). The opiates are potent central nervous system (CNS) depressants but also are stimulants peripherally and centrally (Jaffe and Martin, 1975). Seevers and Woods (1953) postulated that the narcotic analgesics combine with receptors situated at two different sites on the same neuron: on the axon surface and in the cell body. The receptor-drug combination resulted in either depressant (on the axon) or excitant (in the cell body) effects. However, the excitation was normally masked by the depression and was manifested as an abstinence syndrome only after termination of drug administration because tolerance developed more quickly to the depressant effects.

In order for this hypothesis to be feasible there are several requirements: (1) it is necessary for morphine to be present preferentially in nerve tissue throughout the withdrawal period in order to account for the unmasking of the excitant effects - it is not (Seevers and Deneau, 1962); (2) the stimulant properties of the opiates should resemble the abstinence syndrome - this is not the case (please refer to description in Results section - LD50), and (3) physical dependency should be produced by stimulation of the excitant receptors alone. Seevers and Deneau (1962), however, in a critique of their 1953 proposal have shown that physical dependency can be aborted by preventing the depressant actions of morphine with concomittant administration of an

antagonist, suggesting that it may be the depressant properties and not the excitant which are required for the development of dependency.

(iii) Surfeit Theory. Paton explains the effects of morphine-like drugs by their interference at nerve terminals. He suggested that there is a reduction in the release of acetylcholine (ACh) from central and peripheral synapses and that this induces a compensatory reaction which tends to oppose the depression. He proposed that ACh would be dammed back in the axon terminal and the resultant accumulation would eventuate a larger fractional release by each nerve impulse. Hence synaptic transmission is restored to an effective level and a state of tolerance is produced. With the withdrawal of morphine, there is an exaggerated release of transmitter and this excessive stimulation is manifest as the withdrawal syndrome.

There are now considerable data supporting Paton's hypothesis (Beleslin and Polak, 1965; Sharkawi and Schulman, 1969; Jhamandas et al., 1970, 1971) with respect to morphine's ability to impair the release of ACh from nerve terminals. Also, there is much support for the sequestration of ACh within the brain (Giarman and Pepeu, 1962; Collier, 1966; Richter and Goldstein, 1970; Domino and Wilson, 1973), and its explosive release during withdrawal (Crossland and Slater, 1968). This theory has provided a valuable guide for investigators and as a result much information has been acquired on the cholinergic involvement in morphine dependency and withdrawal. Nevertheless, until about 1969, Paton ignored the possibility that there may be coincident changes at the postsynaptic receptor, and that the combination of these phenomena may actually account for most of the reported observations.

(iv) Supersensitivity and Disuse Supersensitivity. Collier's theory (1966) differs from that of Paton in that it explains the development of tolerance and dependence in relation to the action of the drug on tissue receptors rather than on the degree of release from the nerve terminal. He suggested that a drug induces tolerance by changing the number of receptors on which it is able to act and induces dependence by changing the response of the tissue arising from receptor activation. He assumes that there are two types of receptors which can change in number as a result of drug treatment: those that, when occupied, produce a pharmacological response (pharmacological receptors, PR) and those which result in no response (silent receptors, SR). The final, observable response depends upon the proportion of occupied pharmacological receptors. Tolerance results from a decrease in PR or an increase in SR. Dependency and abstinence can be produced by a drug which reduces the supply of endogenous excitatory transmitter in such a way as to increase the PR; upon withdrawal of the drug, supply of transmitter rises faster than the rate of decline in PR. This sudden rise in transmitter level activates the increased number of PR and produces a heightened response - that is, the tissues display supersensitivity.

The "disuse hypothesis" of Jaffe and Sharpless is similar to the theory of Collier in that it postulates a state of supersensitivity (Jaffe and Sharpless, 1968; Sharpless and Jaffe, 1969). This phenomenon is discussed in detail further on in this section (pp 9-16). It differs, though, because they propose that it is the continued depression or disuse of neuronal activity which produces enhanced nerve cell sensitivity. They do not implicate an increased number of receptors. Perhaps a corollary of this theory, although not mentioned,

is that because of the increased sensitivity of a given receptor, there may be a concomittant decrease in its specificity. In other words, in order for the receptor to increase its efficiency, it may become less selective. There is considerable data to support such a "misuse" theory. For example, surgically denervated guinea-pig diaphragm becomes supersensitive to ACh as well as to bradykinin, serotonin and histamine (Alonso de Florida et al., 1965) and rat diaphragm becomes supersensitive to ACh but relaxes in the presence of noradrenaline (Koven and Pinsky, unpublished). One object in this present study was to determine whether there is a generalized increase in sensitivity to differently-mediated CNS-acting tremorigenic agents during morphine dependency, or whether CNS supersensitivity can be demonstrated as specific with respect to individual neurotransmitter systems in the morphine-dependent state.

(C) SUPERSENSITIVITY AND SUBSENSITIVITY

(a) Supersensitivity. This may be defined as "the phenomenon in which the amount of a substance required to produce a given biological response is less than normal" (Fleming et al., 1973). This appears classically as a shift to the left in the dose-response curve, but at times may occur as an increase in the maximum response of a tissue.

Fleming et al.(1973) cite examples of supersensitivity that date back to the 1800's (Budge, 1855). In 1949, Cannon and Rosenblueth stated their "Law of Denervation" which states that the interruption of a functional chain of neurons causes a supersensitivity in all distal elements. They concluded that, because supersensitivity could be induced in a multitude of tissues and by a wide variety of mechanisms,

there must be more than one mechanism involved to account for this generalized phenomenon.

Fleming (1976) has summarized the possible causes for changes in postjunctional sensitivity as: (a) changes in the receptors; (b) changes in the electrical properties of membranes; (c) ionic changes within the cell; (d) biochemical changes; (e) ultrastructural changes. It is clear that one or more of these changes can exist in a given tissue at the same time although their relative importance will be different. With respect to the changes which occur during morphine administration, several of these alternatives have been implicated (Collier, 1968; Gardiner, 1974), although the most common interpretation has been that there are changes in the property or number of receptors.

In a review by Sharpless (1964), a parallel is drawn between peripheral disuse supersensitivity and the centrally-originating phenomena of tolerance and withdrawal. Collier (1966) proposed a similar theory to account for physical dependence, using as his model the alteration in skeletal muscle extrajunctional receptor density during the development of supersensitivity following denervation. He suggested that tolerance and withdrawal are the result of increases in the number of receptors for an excitatory transmitter.

Possible alterations in the number or properties of opiate receptors have been considered in theories to explain opiate dependency. Opiate agonist-antagonist relationships have been studied in whole animals as well as in tissues isolated from opiate-dependent animals. Tulunay and Takemori (1974) investigated the increased effectiveness of the antagonist naloxone, against the analgesic effect of morphine,

in tolerant, non-tolerant, and control animals. They found that the pA2 value, an index of the affinity constant for the antagonist-receptor complex, was considerably increased in tolerant animals. They also treated animals with cycloheximide, a drug that inhibits protein synthesis, and found not only that tolerance did not develop, but also that there was no increase in pA2, suggesting that the synthesis of new opiate receptors may be involved in the observed phenomena. Unfortunately, agents which inhibit protein synthesis do not do so selectively and consequently the animals may be in a compromised state, invalidating the results.

There has been no paucity in the number of neurotransmitters implicated in morphine dependency and in the supersensitivity which develops during repeated administration of morphine. Dopamine and ACh, however, have received the most attention.

Considerable evidence has accumulated on the involvement of the dopaminergic system in morphine actions. Three examples are presented here. Gianutsos et al. (1974) found that there was an enhanced agression in aggregated rats during morphine withdrawal. This aggression could be prevented by morphine and enhanced by apomorphine (a dopaminergic agonist). If the nigrostriatal pathway, a dopaminergic system, was sectioned, aggression was abolished; a low dose of apomorphine reinstated it. Sham lesions and lesions in adjacent areas known to possess tryptaminergic and adrenergic nerve tracts had no effect. The investigators concluded that supersensitivity of dopamine receptors develops during chronic morphine administration. Kuschinsky (1975a, 1975b) however, could not find sufficient evidence to support the conclusion of Gianutsos et al. (1974). He used apomorphine as an indicator of dopamine receptor sensi-

tivity and found that, instead of an increased effectiveness of apomorphine which would suggest supersensitivity of dopaminergic receptors, there was actually a decreased effectiveness. In addition, he records a very interesting observation, that animals, chronically treated with either saline or morphine, will display aggressive behavior. These effects, he concludes, may be attributed solely to the nonspecific stress of injections. The latter statement is contrary to the findings in this laboratory. The animals in the studies presented here were more easily managed and became less aggressive as the injection schedule progressed, suggesting that routine handling decreases the stress of injection.

Cox et al. (1976) also studied the changes in sensitivity to apomorphine during morphine dependency and withdrawal. They found increased stereotyped behavior in morphine-dependent animals and note that this would usually be interpreted as an increase in sensitivity of the receptors. However, they discount this explanation because the increased responsiveness was immediately reversed by naloxone-precipitated withdrawal. How could receptor sensitivity change so rapidly? The results of Gianutsos et al. (1974) were demonstrated in rats undergoing protracted abstinence induced by withholding morphine instead of by injection of naloxone. Their results may reflect residual dependency which persists throughout the protracted course of withholding withdrawal. Cox et al. (1976) provide an alternative explanation based on an interaction between apomorphine and dopamine at the receptor. They suggest that, perhaps, apomorphine is more active than dopamine at the receptor and the apparent sensitivity reflects only the relative concentrations of these two agents at the receptor site. When dopamine

levels are low, as during morphine administration, a large response is seen because more apomorphine can occupy the receptors. During naloxone-precipitated withdrawal, there is an increased dopamine availability. Hence by strict competition, there is less apomorphine occupying receptors and consequently a diminished response.

Isolated tissue and single neuron recording have become popular methods for studying the effects of chronic morphine exposure on various neurotransmitter substances. Yarbrough (1974) investigated the sensitivity of cerebral cortical neurons to ACh and the ability of atropine to block these ACh effects in naive and in morphine-dependent rats. He found that although there was no difference between the two groups in the responses to equal doses of ACh, there was a marked decrease in the ability of atropine to block the ACh-induced increase in discharge rate in morphine-treated animals. These results are difficult to interpret because presumably, both agonist and antagonist are occupying the same receptor sites, yet a state of supersensitivity is consistent only with the data for the antagonist. Frederickson et al. (1975) studied the effects of naloxone and ACh on medial thalamic and cortical neurons in morphine-treated and control animals. Although there were neurons that were more easily excited in the medial thalamus of morphine-treated animals, there was no significant correlation with their sensitivity to ACh. Hence they conclude that the response to naloxone is not mediated directly by ACh. Their results, however, do not agree with those of Satoh et al. (1976), who found a significant lowering of the threshold for increased discharge activity in cortical neurons of morphine-treated animals after both glutamate and ACh. It is difficult to interpret this disparity amongst investigators but undoubtedly the modes of morphine

administration, brain areas sampled, type of anesthetic and quantity of drug applied, are contributing factors. It should be noted, however, that the study by Satoh et al. (1976) was designed to eliminate the variability due to such interfering factors.

Controversy also exists for the presence of supersensitivity in isolated tissues. Muir and Pollock (1973) demonstrated postsynaptic supersensitivity in the rat colon and vas deferens using nerve stimulation in situ and agonists in vitro. They could demonstrate the increased responsiveness to nerve stimulation only upon cessation of morphine administration or by treatment with nalorphine. This latter method is not desirable since nalorphine, a partial antagonist, has effects of its own and consequently may be confounding the results. Because there was increased sensitivity in both these tissues, which represent sympathetically - and parasympathetically - innervated tissues, the authors conclude that it is unlikely that morphine is affecting any specialized processes such as transmitter uptake.

Goldstein and Schulz (1973), using guinea-pig ilea, were unable to demonstrate any change in ACh sensitivity in morphine-implanted animals, even though those ilea were sensitive to morphine's depressant actions on twitch height. Their criterion for tolerance, however, was a diminished hypothermic response to morphine even though it has been shown that tolerance to this effect occurs by the second injection (Lotti et al., 1966). Their animals, therefore, may not have been tolerant as it is usually defined. Shoham and Weinstock (1974) in a carefully controlled study did show supersensitivity to ACh in the acutely tolerant guinea-pig ileum. Strips of ileum were removed from untreated animals and exposed to morphine in an organ bath for 90 minutes. They measured

contraction height and ACh release in tissues exposed to various concentrations of morphine and ACh after this initial perfusion period. They found a reduction in ACh output during morphine perfusion and a diminished efficacy in the ability of morphine to reduce twitch height. This "tolerance", however, was accompanied by a potentiation of the responses to administered ACh, implying the development of supersensitivity. These tissues also exhibited increased sensitivity to histamine and at times to KCl, suggesting that this sensitivity change was nonspecific in nature.

Supersensitivity during morphine treatment has been demonstrated for other than neurotransmitter substances. Gibson and Pollock (1975) were interested in the mechanism of supersensitivity caused by three very different procedures: morphine withdrawal, thyroidectomy and a single dose of reserpine. They used the rat anococcygeus muscle as their test tissue and found that this muscle became supersensitive to ACh and to norepinephrine during all three treatments. Plasma corticosterone levels were elevated with all three treatments as well. Corticosteroids, when administered chronically, produced the same results. Finally, metyrapone, an inhibitor of steroid synthesis, prevented the appearance of supersensitivity during morphine withdrawal. The authors offer no explanation for their observations but speculate that steroids may be acting by altering ionic balances or by inhibiting connective tissue synthesis, thereby modifying the mechanical properties of the muscle and allowing it to develop greater tension.

There is one study which attempts to monitor the behavioural manifestation of changes in neurotransmitter sensitivity. Vasquez et al. (1974), using a fixed reinforcement paradigm, trained rats to press a

bar for water after having been deprived of water for 23 hours. They found that morphine-treated animals, when injected with various cholinergic and adrenergic agents, altered their response rates to a significantly greater degree than did saline-treated animals given the same drugs. These investigators concluded that receptor supersensitivity in more than one pathway could account for their observations.

It will be apparent from the foregoing review that studies done in other laboratories, have examined supersenitivity as an all-or-none phenomenon. There have been no abtempts to monitor its development throughout the time course of morphine dependency. The studies reported here are the first attempt to determine whether it is an early or late phenomenon and whether it is steadily maintained or waxes and wanes during the morphine-dependent state.

(b) Subsensitivity. Just as a diminution of transmitter release results in postjunctional supersensitivity, so can an excess of transmitter produce a state of postjunctional subsensitivity. The first examples of this phenomenon arose from studies on organophosphate cholinestrase inhibitors (Rider et al., 1952; Barnes and Denz, 1954). The investigators noticed a reduced lethality of a given dose of drug with repeated administration. They suggested that their results might be due to an accomodation or adaptation to the increased levels of ACh or perhaps to a plateau of inhibition for the cholinesterase.

An apparent paradoxical subsensitivity has been shown to occur after denervation. Reas and Trendelenburg (1967), in attempting to study denervation supersensitivity in the cat sweat gland, found that the glands became subsensitive to both ACh and pilocarpine during the first two days after surgery. These results, however, are readily explainable by post-

ulating an excessive amount of transmitter being released initially by the degenerating nerve fibres (Emmelin, 1962).

Examples of subsensitivity have also been demonstrated in heart, atrium, ileum, and nictitating membrane (Perrine and McPhillips, 1970; McPhillips, 1969; Trendelenburg et al., 1970). Central cholinergic subsensitivity may be expected during some phase of the morphine abstinence syndrome because of the outpouring of "free" ACh (Crossland, 1970). If (as the model we are using predicts) there is a sudden release of ACh from nerve terminals after removal of morphine blockade, a decreased receptor sensitivity should be demonstrated by the use of cholinergic agonists. In these studies, we have used the tremorigenic efficacy of drugs as a means of measuring receptor sensitivity.

(D) TRANSMITTER AND INORGANIC ION INVOLVEMENT IN MORPHINE DEPENDENCY

Numerous attempts have been made to link the pharmacologic effects of morphine and its derivatives to putative neurotransmitters in the brain. This interaction has implicated neurohormones such as dopamine, norepinephrine, serotonin and ACh. More recently, however, other substances have become somewhat popular subjects for study. They include prostaglandins and cyclic AMP, histamine, calcium and other divalent ions, receptor proteins and most recently, endogenous ligands.

Comprehensive reviews are available for most of the agents mentioned and so any attempt to make an exhaustive review of the entire literature would be redundant and beyond the purpose of this thesis. I have attempted to summarize briefly the major contributions for the more popular candidates and where detailed reviews are lacking, have included more complete information.

(a) Prostaglandins (PG's). The ubiquity of PG's is well recognized.

They have been detected in almost every tissue and body fluid and have been implicated in the responses to a myriad of stimuli. Their involvement in smooth muscle function, gastric and intestinal secretions, kidney function, regulation of the autonomic nervous system, and in peripheral pain is well documented (Horton, 1972). Their central effects, particularly in thermoregulatory control, are also well-established (Feldberg and Saxena, 1971; Veale and Cooper, 1974). That they should be included as a candidate for the mechanism of action of morphine is therefore not surprising.

PG's presumably act via the adenylate cyclase system, and - depending upon the subclass of PG, stimulatory or inhibitory - their effects are correlated with increases or decreases, respectively, in cyclic AMP. The rationale for proposing their involvement in the effects of morphine is based on this very wide distribution of PG's and cyclic AMP, and on the role of the latter as a "second messenger".

Collier (1972b, 1972c) observed that the abstinence syndrome is modified by a wide spectrum of drugs and that even a single drug may have beneficial effects on some signs but detrimental effects on others. He reasoned from these dichotomous results that no single one of the neurotransmitters involved could be playing the primary role in morphine dependency and withdrawal. There has recently been considerable data published on this topic, primarily by Collier's group, which has substantiated the primacy of the PG's and cyclic AMP as mediators of narcotic actions (Puri et al., 1975; Merali et al., 1976; Ho et al., 1975). A review of this evidence is therefore appropriate in this section.

When PGE is added to whole brain homogenate, the yield of cyclic AMP is increased. If morphine also is added to the medium, this PGE

stimulation of cyclic AMP synthesis is inhibited (Collier and Roy, (1974a, 1974b; Van Inwegen et al., 1975). This effect is stereo-specific; levorphanol presents, but dextrorphan has no effect on, cyclic AMP formation. The potencies of various narcotics in this effect are correlated with their relative potencies in analgesic tests (Collier and Roy, 1974b). Moreover, Collier has shown that naloxone, in low concentrations, antagonizes this biochemical effect. At higher concentrations naloxone depressed cyclic AMP formation.

Theophylline, an inhibitor of cyclic AMP phosphodiesterase, prduces a "quasi-morphine abstinence syndrome" which is worsened by naloxone and alleviated by heroin (Collier, 1974; Collier et al., 1974b; Francis et al., 1975). This suggests that elevated levels of cyclic AMP can stimulate morphine withdrawal. Severity of opiate narcotic withdrawal may be altered in a predictable manner by drugs which stimulate (e.g. imidazole) or inhibit (e.g. caffeine) phosphodiesterase. Such drugs must be administered to the morphine - dependent animals just prior to naloxone, and not during chronic administration of opaite.

If cyclic AMP levels are maintained or elevated, severity is enhanced, whereas if the levels are lowered, withdrawal is ameliorated (Collier and Francis, 1975). Similarly, intraventricular injection of cyclic AMP significantly increased severity of withdrawal. In other studies, whole-brain cyclic AMP content correlated with severity of withdrawal in rats (Mehta and Johnson, 1974), and administration of cyclic AMP during dependency intensified abstinence weight loss and decreased the amount of naloxone needed to precipitate jumping. Cyclic AMP did not increase jumping (Ho et al., 1973a) if given only

prior to naloxone, an observation which is in conflict with Collier's data.

Cycloheximide pretreatment resulted in a diminution of the accelerating effect of cyclic AMP on the development of dependency, as was evidenced by the return of the ED50 for naloxone-induced jumping to control levels (Ho et al., 1973). Moreover, cycloheximide alone, when given chronically, prevented the development of physical dependence. This latter observation suggests that cyclic AMP may be involved in the process of protein synthesis (Loh, et al., 1969). The effects of cyclic AMP were specific in that other cyclic nucleotides (2', 3', - cAMP and 3', 5', - cGMP) did not alter the development of dependency, nor did they alter the AD50 (analgesic dose), a measure of tolerance development (Ho et al., 1973).

In an interesting experiment by Collier et al. (1975), brains of morphine-treated and saline controls were submitted to methods of PG extraction. These extracts were then injected intraventricularly into naive rats. Those animals that received extracts from morphine-dependent rats displayed a quasi-abstinence syndrome, whereas those receiving control extracts presented with fewer signs of withdrawal. It was concluded that PG's were elevated in the morphine dependent animals and when transferred to naive animals, produced a simulated morphine abstinence.

If one examines some of the individual effects of morphine and compares the direction of these to the direction of effects of cyclic AMP and PG's, a reasonably good inverse relationship emerges. Morphine elicits running in the mouse (Goldstein and Sheehan, 1969; Jaffe and Martin, 1975), constipation and contraction of the sphincter of Oddi, whereas PG's

and cyclic AMP produce sedation in the mouse (Horton, 1972), diarrhea (Misiewicz, 1969) and relaxation of the sphincter of Oddi (Anddrsson, 1973). Cyclic AMP antagonizes Straub-tail in mice and running elicited by morphine (Loh, 1971); morphine suppresses the nociception produced by intraperitoneal injection of PGE in mice (Collier, 1972). Such examples of an antagonism between the pharmacological effects of these substances provide good indirect evidence of a possible competition at the receptor level. However, data for the stimulation of PG synthesis by morphine (Collier et al., 1974a) seem to, at first, contradict the feasibility of such an interaction. Why should a drug, morphine, which has been shown to cause effects opposite to those of PG's defeat its purpose by increasing the synthesis of its competitor? Teleologically this appears unsound. Nevertheless, morphine possesses several stimulant effects such as emesis, hyperthermia and hyperglycemia, and they may be related to the drug's enhancement of PG biosynthesis. Hence, this dual action of morphine may account for the biphasic effects of morphine, and for some of the interactions with neurotransmitters.

(b) Norephinephrine (NE). Extensive attempts have been made to implicate NE in the acute and chronic effects of morphine. Results from such studies have, on occasion, been so contradictory as to make it appear that each successive publication has increased confusion in this area instead of contributing clarification thereto.

Depending upon the species, the route of drug administration, dose, and number of injections, the content of brain catecholamines has been reported to increase, decrease, or not change, following morphine administration (Vogt, 1954; Gunne, 1963; Maynert and Klingman, 1962; Segal et al., 1972). As brain levels of NE represent only gross changes, more

recent attempts at evaluating NE's role have concentrated on measurements of turnover in whole brain and within localized areas of brain. These studies have used the incorporation of ^{14}C-tyrosine into ^{14}C-NE and/or ^{14}C-dopamine as an index of biosynthesis. Clouet and Ratner (1970) and Johnson et al., (1974) found that morphine increases the conversion of ^{14}C-tyrosine into NE and tolerance to this effect fails to develop. The acute effects were confirmed by Smith et al. (1970, 1972) and Sheldon et al. (1975), but these chronic results are in conflict with those of Smith et al. (1970, 1972) and Rosenmen and Smith (1972), who found that tolerance to this effect does indeed occur. They found that once tolerance to a behavioural effect, such as analgesia, is established, mouse brain cannot maintain normal rates of synthesis without repeated morphine administration. Furthermore, during withdrawal, as the brain regains its ability to synthesize NE, an injection of morphine becomes more effective in once again increasing the synthesis of NE.

A slightly different approach to measuring NE turnover was taken by Roffman et al. (1975). They measured the levels of the sulfate conjugate of 3-methoxy-4-hydroxphenylglycol (MHPG), the major metabolite of NE in rat brain (Schanberg et al., 1968). They found that acute injections of morphine produced dose-related increases in the levels of MHPG. Chronic treatment resulted in lower baseline levels of MHPG and tolerance to its acute effects. These results confirm those of Smith's group, that maintenance of catecholamine synthesis requires continued morphine administration.

Despite these biochemical observations, the current understanding of the role of NE in morphine dependency, as determined by methods which either increase or decrease NE brain levels, is in a chaotic state. For

instance, Friedler et al.(1972) found that pretreatment with 6-hydroxydopamine (6-OHDA) reduced morphine analgesia and enhanced withdrawal jumping in mice. Brain NE levels were reduced by 66%. In contrast, Blasig et al. (1975) found no change in the severity of withdrawal in rats treated similarly with 6-OHDA. Pretreatment with alpha-methyltyrosine (AMT), an inhibitor of the rate-limiting enzyme of NE snythesis, tyrosine-hydroxylase, did reduce the severity (Blasig et al., 1975; Glick et al., 1973). Herz et al. (1974) noted a shift in the withdrawal signs displayed after pretreatment with AMT with a decrease in the overall severity. Selective inhibition of NE synthesis by FLA-63 also decreased severity, indicating that NE and not dopamine was primarily responsible for dependency. Also, desipramine, which blocks NE uptake and therefore increases synaptic levels of NE increased the severity of withdrawal.

Based on studies indicating that alpha-adrenergic blockers such as chlorpromazine and phenoxybenzamine possess analgesic properties (Cicero et al., 1973), increase the magnitude and duration of morphine-induced analgesia, and enhance morphine lethality, attempts were made to modify the withdrawal syndrome using these agents (Cicero et al., 1974). They found that propranolol, a beta-adrenergic blocker, failed to modify the withdrawal syndrome, whereas alpha-adrenergic blockers caused a dose-dependent decrease in the amount of diarrhea and in the number of wet-dog shakes. Chipkin et al. (1975) confirmed the lack of effects of propranolol but Bhargava et al. (1972) found that dichloroisoproterenol, another beta-blocker, ameliorated the withdrawal syndrome.

Human studies by Grosz (1972, 1973) and Jacob et al. (1975) have suggested the propranolol may have beneficial effects in the initial de-

toxification period. Others, however, have disputed this. Resnick et al. (1976) has claimed, "propranolol neither relieved nor precipitated opiate withdrawal", and Matas (unpublished), has described a toxic deliriant psychosis provoked by the administration of propranolol to a heroin-dependent subject undergoing withdrawal. Thus, there is scant experimental data to suggest a primary role for beta-adrenergic receptors in morphine dependency. Clinical trials, albeit poorly controlled, suggest that they might be involved in the withdrawal state, perhaps in some secondary fashion. Manipulation of central beta-adrenergic transmission, either during the development of dependency, or during withdrawal, seems not to be a promising approach either in preventing opiate readministration or in relieving the withdrawal sickness.

(c) 5-Hydroxytryptamine (5-HT; Serotonin). There is considerable controversy over the role of 5-HT in the morphine tolerant-dependent state. The major proponents of the importance of 5-HT in morphine's effects have been Way and his co-workers who have published numerous articles on the subject, many of which have been repetitious, in order to refute the criticism of other investigators who could not confirm their results.

Way et al. made their initial report in 1968, stating that the rate of 5-HT synthesis increased during morphine administration, and that this correlated with the development of tolerance, as measured by an increase in the amount of morphine required to produce analgesia (AD50). The turnover of 5-HT was measured by inhibiting metabolism of 5-HT to its metabolite 5-hydroxyindole acetic acid (5-HIAA) with pargyline, a monamine oxidase inhibitor. Similarly, parachlrophenylalanine (PCPA), an inhibitor of 5-HT synthesis, increased the AD50 in morphine-treated

animals and diminished withdrawal jumping.

These results were in conflict with those of Maynert et al. (1962a, 1962b), Sloan et al. (1963), and of Cochin and Axelrod (1959), who found that levels of 5-HT were not altered by morphine. Way et al. (1968) discount this conflict by the fact that his group measured turnover and not steady state levels, as was done by others. Nevertheless, this disagreement amongst investigators continued even when others attempted to use similar experimental protocols (Marshall and Graham - Smith 1971; Cheney et al., 1971; Maruyama et al., 1970; Schwartz and Eidelberg, 1970; Algeri and Costa, 1971). In general, those investigators have found that turnover of 5-HT is not increased during morphine dependency, and that when synthesis is decreased by PCPA, the withdrawal syndrome is not altered. Way and his colleagues have continued to publish results to the contrary (Way et al., 1968; Way 1972, 1973; Shen et al., 1970; Loh et al., 1969) and have found support from others (Haubrich and Blake, 1969; Tilson and Rech, 1974; Papeschi et al., 1974). The discrepancy amongst authors is difficult to explain because attempts were made to control differences in technique and species. These variables, therefore, may be excluded as possible factors contributing to the lack of agreement amongst investigators.

It is readily apparent that the role of 5-HT in the chronic effects of morphine is, at best, questionable. This putative neurotransmitter may be involved in some of the acute effects of morphine such as hyperthermia (Warwick et al., 1973; Fuller and Baker, 1974) and stereotypy/catalepsy (Costall and Naylor, 1975), but it appears unlikely to be primarily involved in the phenomena of tolerance and dependence.

(d) Dopamine. This neurotransmitter has become increasingly implicated, over the past five or six years, in the mechanism of action of morphine.

The naroctic withdrawal syndrome includes among its many behavioural characteristics, those of hyperirritability to environmental stimuli (Puri and Lal, 1974) and aggressiveness (Lal, 1975). Withdrawal aggression has been documented for the rat (Lal, 1975), mouse (Weisman, 1973), and guinea-pig (Goldstein and Schulz, 1973). Study of this latter sign was the first line of evidence for a postulated involvement of the dopaminergic system, or more exactly, of dopamine-receptor supersensitivity in narcotic dependence. This withdrawal aggression (Lal et al., 1971; Puri et al., 1971) is enhanced by directly and indirectly acting dopamine-receptor agonists such as DOPA, amphetamines or apomorphine (Puri and Lal, 1973). The aggression is prevented by narcotic administration (Gianutsos et al., 1974), by dopamine-receptor blockers such as haloperidol (Puri and Lal, 1973), or by lesioning the nigrostriatal bundle (Gianutsos et al., 1974), a dopaminergic nerve tract. AMP prevents the effect of amphetamines which act indirectly via release of central catecholamines, but not the effects of apomorphine, a directly-acting dopamine agonist(Puri and Lal, 1973). This indicates that a state of receptor supersensitivity may be present during morphine administration.

Withdrawal jumping in mice has been correlated with changes in brain dopamine concentration (Iwamoto et al., 1973), and this jumping is potentiated by low doses of apomorphine (Herz et al., 1974). Also, jumping which can be produced by a combination of l-DOPA and amphetamine in naive mice, can be blocked by an injection of morphine sulphate (Lal et al., 1975). It should be noted, however, that withdrawal jumping can

also be prevented by previous administration of cholinergic agents (Iwamoto et al., 1973; Jhamandas and Sutak, 1974), suggesting and interaction between cholinergic and dopaminergic systems in the brain. Moreover, recent studies utilizing various mouse strains have indicated that there is no correlation between steady-state brain dopamine levels and naloxone precipitated withdrawal jumping (Reinhard et al., 1976). Consequently, the results of Iwamoto et al. (1973) may have occurred by chance, in that the strain of mice used in their studies may be one whose dopamine levels are modified during withdrawal jumping.

Biochemical studies reveal an increased turnover of striatal dopamine by acutely administered morphine. Increased synthesis is evidenced by an increased conversion of tyrosine to dopamine (Smith et al., 1972) and an accumulation of DOPA after inhibition of brain DOPA decarboxylase (Gauchy et al., 1973; Sugrue, 1974). Similarly, the metabolite of dopamine, homovanillic acid, increases in amount, suggesting an increased dopamine catabolism (Kuschinsky, 1973). These effects were all prevented by administration of naloxone (Sugrue, 1974). It must be emphasized that all these results give indirect evidence, and that there is little evidence for narcotic interaction with dopamine receptors. Nevertheless, general agreement exists amongst investigators that dopamine is implicated in the mechanism of actions of morphine. Whether there is a causal relationship between morphine's effects and brain dopamine levels remains to be ascertained.

(e) Acetylcholine (ACh). ACh is synthesized from choline and acetyl-CoA in a reaction that is catalyzed by choline acetyl transferase (Nachmansohn and Mackado, 1943; Hebb, 1972; Hubbard, 1973; Hrdina, 1974). Choline, despite its quaternary structure, passes the blood brain barrier

(Schuberth et al., 1969, 1970; Dross and Kewitz, 1972) and enters cells via a transport process (Martin, 1968; Adamic, 1970; Diamond, 1971).

It has been recently demonstrated that choline may regulate the synthesis of brain ACh since the latter process can be stimulated in vivo by elevating the tissue concentration of its precursor (Haubrich et al., 1975; Cohen and Wurtman, 1975). These results suggest that, the levels of free choline in brain are less than those required for maximal ACh synthesis. In addition to its role as a precursor to ACh, choline has weak cholinergic activity (Burgen and Mitchell, 1968; Sanyal et al., 1970) and behaves like a partial agonist (Stephenson, 1956) on cholinergic receptors (Frederickson, 1971).

It is well known that morphine reduces ACh release from peripheral cholinergic structures (Paton 1957, 1963; Schaumann, 1957; Cox and Weinstock, 1966; Kennedy and West 1967; Frederickson and Pinsky, 1971, Pinsky and Frederickson, 1971; Lees et al., 1972). Also, narcotic analgesics reduce the release of ACh from rat brain (Jhamandas and Sutak, 1974; Matthews et al., 1973), rabbits (Beleslin and Polak, 1965; Beleslin et al., 1965), and cats (Jhamandas et al., 1970, 1971). However, administration of morphine reduces the extractable "free" ACh content of brain (Crossland and Slater, 1968), thereby implying a sequestering of ACh within nerve terminals.

The "running fit" in mice has been used as an index for measurement of tolerance to opiates (Goldstein and Sheehan 1969). Levorphanol produces this stereotyped locomotor activity in mice; it is easily measured with photoelectric counters. Sharkawi and Goldstein (1969) examined the possibility that a central cholinergic system is involved in the running fit. They administered the acetylcholinesterase inhibitor, phy-

sostigmine, to protect ACh and thereby antagonize the anti-ACh release effects of morphine. Their results confirmed this possibility over a wide dose range of levorphanol.

Richter and Goldstein (1970) found increases in "free" and "bound" ACh in mouse and rat brain after administration of morphine or levorphanol, but not after dextrorphan (the inert stereoisomer of levorphanol. Their results agree partly with those of Crossland and Slater (1968) who found increases in the "bound" fraction but a decrease in the "free" ACh. That the "free" fraction is indeed not an artifact, is supported by Sharkawi's data (1972) which indicate that labile (cytoplasmic) and stable (vesicular) fractions of bound ACh, constitute only 70 to 90% of total ACh. In all likelihood it represents a mixture of ACh derived from newly synthesized cytoplasmic ACh, released transmitter, and some bound ACh released by the extraction process. Large and Milton (1970) found that whereas a single-dose of morphine caused an increase in rat brain ACh, this effect was absent in chronically-treated animals. They also found a rise in brain ACh after abrupt withdrawal of morphine, which reached a peak at 39 to 46 hours post-withholding, or abruptly after a precipitated abstinence syndrome provoked by nalorphine. Crossland (1970) found similar results in the rat, but stated the abstinence syndrome is characterized by an explosive increase in the amount of "free" ACh.

Domino and Wilson (1973a) reasoned that, if morphine prevents ACh release, it should reduce the rate of ACh depletion after inhibition of its production by hemicholinium. A similar reduction was to be expected for the depletion produced by the release-enhancing effects of scopolamine. They measured the effects of morphine on brain ACh depletion that had been induced either

by intraventricular hemicholinium and after scopolamine. Their results indicated that morphine prevents depletion of brain ACh regardless of the depletor used. In addition, narcotics from other classes, such as meperidine, codeine, methadone and levorphanol all had similar actions. They suggested that this is a common mechanism of narcotic action and that their anti-depleting activity is related to their analgesic potency.

In the foregoing discussion, evidence has been provided for a sequestering effect of morphine on ACh. The result of this action would be diminished ACh release, with enhanced presynaptic levels of ACh. Acutely, this would cause decreased receptor occupancy. With maintained morphine administration, a compensation may result wherein postsynaptic receptors become more sensitive and therefore, the "effector" response is observed as normal. The ever-increasing requirement for morphine, to achieve the same effects as seen initially, may be thus explained by this postsynaptic compensation.

Presynaptic sequestration of ACh may have consequences seen during withdrawal from morphine. To account for the signs observed in the morphine withdrawal syndrome, Paton (1963, 1969) and Collier (1968) postulated that the sudden removal of the morphine blockade results in an excess of ACh falling upon receptors that had become supersensitive.

Hence, in the development of morphine dependency, cholinergic drive may be in a marginal state, whereas in morphine withdrawal, cholinergic drive is great. A moderation of these two extremes should alter the final outcome of repeated morphine administration; i.e. an increase in cholinergic stimulation during the development of morphine administration with a suppression of central cholinergic stimulation during withdrawal,

should ameliorate the severity of the abstinence syndrome.

One method of accomplishing this is by the use of a partial cholinergic agonist. Not only will choline chloride moderate cholinergic drive through its occupancy of receptor sites but it will also increase ACh synthesis via its precursor function. Thus, the combined effects of choline chloride should to some extent overcome the anti-release mechanism of morphine, prevent the development of supersensitivity, and diminish the exaggerated cholinergic stimulation seen in the withdrawal syndrome (Frederickson and Pinsky, 1975).

The abstinence syndrome has classically been evaluated by a constellation of signs (Himmelsbach, 1935, 1943; Way et al., 1969; Frederickson, 1971), each being assigned a numerical value as a function of its severity, and subject to considerable experimenter error. A more quantitative measure is the assessment of jumping behaviour, as in rodents this is an all-or-none event (Huidobro and Maggiolo, 1961; Kaneto and Nakanishi, 1971; Way et al., 1969; Collier et al., 1972; Marshall and Weinstock, 1971) which can be reliably measured by different experimenters. In the studies presented here, the latter method was used primarily, but the classical approach was also used on occasion, in an attempt to verify the beneficial effect of choline chloride on the morphine abstinence syndrome.

(f) Calcium Ion. A number of reports have indicated that narcotic drugs alter the levels of brain inorganic cations, in particular, calcium. Kakunaga et al. (1966) reported that intracisternal administration of calcium ion suppressed, in a dose-related manner, the analgesia produced by narcotics. Calcium chelators, while having no effect alone, enhance the analgesia rpoduced by morphine. This antagonistic effect

appeared to be unique to calcium, as other cations were virtually ineffective. The shifts in dose-response curves were parallel, perhaps indicating competitive antagonism (Harris et al., 1975). Intracerebroventricularly administered lanthanum, had antinociceptive effects which were antagonized by peripheral administration of naloxone or by intraventricular calcium. Animals tolerant to morphine were also found to be tolerant to lanthanum analgesia (Harris et al., 1976). Since lanthanum is known to inhibit calcium binding and movement, these data suggest that analgesia may be related to decreased binding of calcium at certain critical sites on the affected neuronal membrane. Finally, the ionophore X537A, which increases membrane permeability to divalent cations, antagonized the analgesic effect of morphine when administered in conjunction with low doses of calcium, an effect not seen with either alone (Harris et al., 1975). This implies that it is an increased calcium flux across neuronal membranes which inhibits morphine's actions, and that morphine might act by inhibiting calcium movement.

Studies on brain calcium changes induced by morphine-like drugs have shown a dose-related depletion of calcium by morphine (Ross et al., 1974), an effect that is also seen with levorphanol, but not its inactive isomer, dextrorphan (Cardenas and Ross, 1975). Naloxone, by itself, had no effect on brain calcium but blocked the morphine-induced depletion (ibid; Ross et al., 1976). Evidence of this nature, showing a response having opiate stereospecificity and reversibility by naloxone, has been argued to be major criteria for implicating the involvement of a particular brain substituent in the mechanism of opiate action. Thus, calcium, known to be involved in a variety of other biochemical

processes within the body, also satisfies the criteria for a role in morphine actions.

A recent report has indicated that chronic morphine administration also reduces brain calcium (Sanghvi and Gershon, 1976). Prior intraperitoneal administration of calcium in the morphine implanted group, significantly reduced naloxone-precipitated abstinence signs. This confirms an old report by Detrick and Thienes (1941) that a high-calcium diet plus parathyroid hormone, weakened the withdrawal symptoms compared to rats on a low calcium diet.

(E) CHOLINE TOXICITY AND USEFULNESS OF CHOLINE IN MORPHINE DEPENDENCY

The acute and chronic toxicity of choline in rats was studied by Hodge some thirty years ago (1944, 1945). He found that the LD50 (i.p.) values changed as a function of the concentration of drug administered. His values ranged from 370 mg kg^{-1} at a concentration of 200 mg ml^{-1}, to approximately 670 mg kg^{-1} at a concentration of 20 mg ml^{-1}. The lethal effect was extremely rapid and death was preceded by such signs as trembling, convulsions, salivation, red lacrimation (chromodacryorrhea) and changes in respiration. Animals that survived for 20 minutes invariably did not succumb as a result of the choline given. Rats that were given dietary choline in amounts of 0.1% or 1.0% of the total diet did not differ from control animals in weight over a period of 3 months. In addition, no histopathological changes were observed in the dietary studies.

Many of the experiments reported here were done on mice and consequently we felt it necessary to determine the acute toxicity of choline in that species. In addition, short term cumulative effects were examined.

Biological clocks within living organisms are now considered to be major determinants of behavioral and physiological rhythms (Rusak and Zucker, 1975). Also, reponsiveness to drugs varies with the phase of the organism's circadian cycle. Lutsch and Morris (1972) varied lighting conditions and examined the changes in morphine analgesia that resulted. Under standard lighting conditions, peak analgesia, measured by the Haffner tail-pinch technique, occurred in the dark period, with a trough in the light period. These results were supported by Bornschein et al. (1973). When the light-dark schedule was reversed, there was a reversal in the temporal pattern. Exposure to constant lighting produced a more uniform analgesic response.

Using the LD50 for methadone, Lenox and Frozier (1972) found that the number of deaths fluctuated as a function of time of day. The animals were most sensitive in the period of transition from rest to activity, and most resistant when they were nearing their activity period. The authors pointed out the importance of these findings to the maintenance of heroin addicts who receive several days supply of methadone at one time.

Ayhan (1974) demonstrated a diurnal sensitivity to morphine-induced hyperactivity in rats. There was a significant increase in activity produced by morphine during the middle of the dark period with least activity just prior to the light period. Although transmitter measurements were not made, Ayhan referred to the daily fluctuations known for the catecholamines and suggested that the two might be casually related.

Bornschein et al. (1973), using doses of morphine that caused deaths in mice by either excitation or depression, examined the proportion of animals that died in each category as a function of time of

day. They found that mice were maximally sensitive to the depressant actions of morphine during the middle of the dark period and maximally sensitive to the excitatory effects early in the light period.

Hannin et al. (1970) measured ACh concentrations in rat brain over a 24 hour period and found that there was a peak at 2 hours into a 12 hour light cycle and a trough 6 hours into the dark cycle. This pattern emerged only after 18 days of adaptation to this 12 hour light-12 hour dark cycle and occurred only in grouped animals. Animals that were housed individually displayed no differences in ACh levels. These results differ from those of Walker and Chaichareon (1973) and of Friedman and Walker (1972). Both groups found peak ACh levels during the dark phase although they did not mention whether animals were housed as groups or individually. Friedman and Walker (1972) also found that maximum toxicity of neostigmine, pilocarpine and oxotremorine occurred during the dark phase. The opposite results were found for atropine.

Thus there is an abundance of data supporting the importance of the time of drug administration, in the physiological and behavioural parameters measured. The necessity of performing experiments at the same time of day is therefore obvious. Finally, the potential significance of circadian rhythm on the cumulative toxicity of cholinergic agents has been examined in our studies.

(F) THE ROLE OF AN ENDOGENOUS OPIATELIKE FACTOR IN MORPHINE'S ACTIONS AND IN MORPHINE DEPENDENCY

Near the completion of the research on tremor and its modification by morphine administration, reports dealing with the characterization of opiate receptors began to appear in the literature.

Several aspects of opiate narcotic action prompted investigators

to consider the possibility that opiates and opioid drugs were interacting with highly selective and specific sites within the brain. Evidence in support of this concept is summarized in the following considerations: (1) opiates, in general, conform to a particular chemical structure; (2) some potent opiates such as etorphine exert their effects in extremely small doses; (3) stereospecificity is important for opiate actions, the levorotatory (-) isomer being the active form; and (4) naroctic antagonists can prevent opiate effects without themselves possessing agonist actions.

The identification of highly stereospecific opiate binding to brain tissue was first suggested by Goldstein (1971) and confirmed thereafter in work reported independently by Pert and Snyder (1973), Simon (1973), and by Terenius (1973). This has now been confirmed in many laboratories (Hitzemann and Loh 1973; Wong and Horng, 1973; Lee et al., 1973; Klee and Streaty, 1974). There is a close parallel between pharmacologic potency and affinity for the opiate binding sites.

The distribution of opiate binding sites within the brain is uneven. The area of highest binding is that region defined as the limbic system (Kuhar et al., 1973; Hiller et al., 1973). This agrees with results from electrical stimulation (Borison, 1971) and ablation studies (Kerr and Pozeulo, 1971) which indicate that these areas are important for opiate actions. The subcellular distribution is primarily restricted to the synaptic membrane fraction (Pert et al., 1974).

Perhaps the most important contribution was made by Pert and Synder (1974) who demonstrated that sodium enhances antagonist binding. They concluded (1975), along with Simon (1976), that the sodium ion acts as an allosteric effector which produces a conformational change in the

opiate binding site. The presence of sodium increases the affinity for antagonists while decreasing affinity for agonists, whereas the absence of sodium has the opposite effect. Furthermore, Pasternak and Snyder (1975) demonstrated that there were two binding sites for naloxone with different affinities, and that sodium affected only the high-affinity binding site.

In an address to the Pharmacological Society of Canada (1974), Goldstein speculated on the existence of endogenous opiate-like compounds based on the then revolutionary discovery of opiate-receptors. Why should there exist such specific binding sites if they were not meant as targets for endogenous substances? Frederickson (1977) has noted that this concept had been suggested previously (Martin, 1967; Collier, 1971, 1972a), but it was Goldstein's comments that prompted us to consider this issue further. The connection between this and our studies on cholinergic function in opiate responses was seen to reside in the cogent possibilities that the actions of opiates on central ACh function might either be mediated by stereospecific opiate receptors located at cholinergic synapses, or that cholinergic transmission in the brain (especially in the limbic and neostriatal systems) might normally be interacting with endogenous ligands for brain opiate receptors. Hence, the cholinergic upset we had discerned in experimental morphine dependency might have been due at least in part, to the effects of opiates on kinetics of an endogenous morphine-like substance, and on the interaction of this putative "morphine-like factor" with its target receptors in the CNS.

It is important to emphasize the timing of this research as related to the explosion of reports that occurred concerning endogenous ligands.

The results of our research were presented at the same meeting that witnessed the presentation of much of the first work done on enkephalin pentapeptides. There had only been two or three papers published in this field at that time (Hughes, 1975; Terenius and Wahlstrom, 1975). Consequently, our approach to this problem may be considered unique. Much data, using our methods, has been accumulated since our first investigations.

One of the roles we postulated for endogenous opiates was their participation in chronically maintained pain or noxious stimuli. To test this hypothesis we required a test which would produce a low level of discomfort to the animal, in order to induce the release of these endogenous substances. The tail-flick analgesia test and its like, were not suitable as they measured the response to an acute stimulus. Consequently, we decided to use a warmplate method, whereby we could determine an optimal temperature at which the animals would show signs of some discomfort, but yet could tolerate extended periods of observation without obvious injury or distress. This is described more fully under the Methods section.

There are many similarities between steroid and opiate effects and actions (LaBella, 1975, and personal communication). Included in these are: structural resemblance, acute and long-lasting effects, withdrawal syndrome, and brain localization. We considered, therefore, that steroids might be serving as endogenous opioid substances. Hence, we did experiments designed to test the role of endogenous steroids in morphine antinociception tested during a sustained mildly noxious stimulus (Pinsky, Koven and LaBella, 1975). Moreover, we tested the hypothesis that an endogenous morphine-like substance, of then-unspecified chemical nature,

might be revealed behaviourally by an interaction with the relatively pure opiate antagonist naloxone (Fig. 29). To our best knowledge, these concepts were tested experimentally in this study before any hint of a similar approach had appeared in the literature and were independently coincident with the earliest work done elsewhere.

(G) INJECTION TECHNIQUE vs PELLET IMPLANTATION FOR INDUCING DEPENDENCY

Collier (1972a, 1972b), and his colleagues (Collier et al., 1972) hold that morphine dependency is a multipartite phenomenon. In their view the differences amongst investigators as to which neurotransmitter is mainly responsible can be attributed to this multipartite nature. They argue that various drugs have differential effects on the severity of different withdrawal signs when given during dependency or just prior to abstinence. Their own results indicate that atropine, PCPA and indomethacin, which alter the levels of ACh, 5-HT and prostaglandins, respectively, will appear to lessen or worsen the severity of the withdrawal syndrome depending upon the index measured and the time of administration.

Undoubtedly another contributing factor is the method used to cause dependency. Although total morphine exposure, as measured by the area under the plasma level vs time curve, is the same for pellet-implanted or morphine-injected animals, peak concentrations are 3-or-4 fold higher for the latter group (Cerletti et al., 1975). The two different methods of chronic administration may result in equal amounts of naloxone-precipitated jumping, given some appropriate choice of morphine dosages. However, drugs given to modify the withdrawal reaction may have quite different interactions with neurotransmitters in the presence of the differing morphine levels.

Domino and Wilson (1975) measured brain ACh turnover in morphine pellet-implanted rats and compared these results with those acquired from a study using the injection method (Domino and Wilson, 1973). The results from the two studies were virtually opposite to each other. The investigators concluded that "chronic exposure to a narcotic agonist undoubtedly causes biochemical and physiological changes that do not have time to occur in the 3-day morphine pellet rats", and that "it is not valid to make comparisons between two modes of drug administration when drug dosages, brain levels and time courses are not comparable".

There is little doubt that dependency on morphine as produced by pellet implantation for 72 hours is more economical than that produced by twice-daily injections over a period of weeks. The studies just cited, however, show why there is considerable controversy as to the validity of the former model in terms of the changes that can occur in such a short-term exposure, as compared to the chronic exposure that is achieved by the injection technique. Undoubtedly, many of the apparent contradictions that exist in the literature have resulted from this obvious difference in methodology. It is imperative, therefore, that great care be taken in the interpretation of results that have been acquired using these two very different modes of morphine administration.

II METHODS

(A) CHOLINE-MORPHINE STUDIES

(a) Antagonist-precipitated jumping in morphine-treated mice. Female albino mice (CD^R-1 Strain, Canadian Breeding Laboratories, Montreal) initially 18-23 gm were assigned randomly to six different treatment groups (gps) housed in sawdust-bedded plastic cages, four per cage. Animals were acclimated for several days to laboratory conditions, 25°C, relative humidity 30%. A 12hr light - 12hr dark cycle was used with food and water ad libitum. Drugs were administered intraperitoneally (i.p.) in such concentrations that all animals received equal volumes of drug or saline solutions per body weight.

Animals were classified as "jumpers" if they leaped, with four feet simultaneously off the bottom of a glass observation jar (30 x 30 x 60 cm), at least once over a 10-min observation period. Analysis of differences in the proportion of jumpers in each group was done for a two-tailed distribution according to the method of Hoel (1960). Differences which yielded a value of $p < 0.05$ were taken as being significant.

(b) Effects of choline on morphine withdrawal in mice.

(i) Habituation to morphine. Morphine sulphate (MS; British Drug House) was administered i.p. by single daily injections to 4 groups (Gps 1,2,3 and 6; regimen MS-1) of mice starting at a dose of 10 mg kg^{-1} with daily increments of 40 mg kg^{-1} to a final dose of 330 mg kg^{-1} on da 9. Two other groups (Gps 4 and 5) were given the same total daily dose, divided into two equal doses (morning-evening; regimen MS-2) at 12 hr intervals. Animals were challenged with nalophine hydrochloride 10 mg kg^{-1} or with an equivalent volume of saline. They were then observed for abstinence jumping, as described previously.

(ii) Choline treatment. Gps 1-5 of the six morphine-treated groups received choline chloride (Sigma) i.p., each according to one of four schedules (regimens C-1, C-2, C-3, and C-4; Table I) 15 min after MS injection. Gp 6 of the morphine-treated mice received saline injection in lieu of choline.

(c) Effects of choline on morphine withdrawal in rats. Young adult male Sprague Dawley rats, initially weighing 60-80 gm, were used throughout. The rats were maintained in constant environmental conditions and were allowed water and food ad libitum. Several days were allowed for acclimation to this environment before commencement of the habituation schedules. All animals were tested in an adjoining room.

In all cases, the drug concentrations of MS and choline chloride were adjusted in order to increase the dosage continuously while maintaining a constant injection volume.

Rats were assigned randomly to treatment groups. All morphine-habituated animals were made tolerant to at least 300 mg kg^{-1} MS twice daily. Animals received injections i.p. twice daily of gradually increasing quantities of MS. Doses began with 10 mg kg^{-1} given twice daily and increased to 300 mg kg^{-1} twice daily, by da 15. Solutions were made in such concentrations that no animal received in excess of 1 ml for every 100 gm of body weight per injection. Gp 1, designated as MS-N, were morphine-habituated and were challenged with either nalorphine or naloxone. Gp 2 designated MC-N were morphine-habituated but were given choline chloride during their habituation schedule. This group was challenged as in Gp 1. The following groups were controls: Gp 3, designated SS-N, given saline in lieu of morphine and then given antagonist; Gp 4 designated as SS-S, given saline in lieu of morphine and saline in lieu

TABLE I

CHOLINE DOSE SCHEDULES
mg kg^{-1}

	C-1	C-2	C-3	C-4	no choline
Group*	1	2	3	4 and 5	6
DAY 1	2.5	10	10	10	saline
2	5.0	20	20	20	"
3	10.0	40	40	40	"
4	15.0	60	60	2 x 30	"
5	20.0	80	2 x 50	2 x 40	"
6	25.0	100	2 x 70	2 x 60	"
7	30.0	120	2 x 80	2 x 70	"
8	35.0	140	2 x 100	2 x 80	"
9	40.0	150	2 x 120	2 x 80	"

* see METHODS; p.41 "Habituation to morphine".

of antagonist; Gp 5, designated as MS-S were morphine habituated but were given saline in lieu of antagonist and Gp 6, designated as SC-N received saline and choline during the habituation schedule; they then received the antagonist as in Gp 1.

All rats were placed on a 4" by 5" platform immediately after the injection at the time of the experiment. The platform projected from a pool of water in a laboratory sink, thus providing a threatening stimulus to the animals. In addition, all animals were observed for other signs of withdrawal.

To analyze differences in the proportions of leapers, the Yates Correction was applied in the $\bar{X}^2$ test. The data for the number of jumps per animal were transformed by the square root method because of the high frequency of zeros and low numbers. These values were then analyzed by a t-test. In all cases, only those differences at the $p < 0.05$ level or better were considered to be significant.

(d) LD50 of morphine in rats. Background: Tatum (1929) found that monkeys given moderate doses of morphine (50 - 150 mg kg^{-1}) died exhibiting signs of depression. After much larger doses of morphine (300-500 mg kg^{-1}) monkeys died showing signs of excitation such as convulsions and exhaustion. Interestingly, intermediate doses caused no alarming effects. These results prompted Tatum to propose the dual-action theory (discussed earlier on pp6-7) which was later modified by Seevers and Deneau (1962).

We became interested in morphine lethality for several reasons. We wished to attain a high degree of dependence in our animals so that the withdrawal syndrome would be accordingly severe. This was necessary because some of our manipulations in these animals may have produced

only minor beneficial effects and unless the withdrawal syndrome was severe we may not have been able to detect these differences. Consequently, we required some information about the upper limits of non-lethal doses of morphine.

Secondly, in order that we could state that our animals were indeed tolerant to these high doses and that at these doses, the animals were not experiencing the undesirable effects of high doses given acutely, we had to demonstrate acute effects for comparative purposes.

Sprague Dawley rats of 100-120 gm were allowed several days of acclimation to the housing facilities prior to these experiments. All animals were housed individually in wire cages in order that they could not suffocate in the sawdust bedding after morphine injection. Injections were made in a volume of 1 ml 100 gm^{-1} and concentrations were adjusted accordingly. Seven groups of 12 animals each received doses ranging from 200 to 1000 mg kg^{-1}. Animals were observed continuously over a 4 hr period and the times of deaths were recorded. The number of deaths after 1 and 2 hr were used for determination of LD50 by means of a reiterative regression-line approximation computer program.

(e) Determination of choline toxicity.

(i) Acute toxicity. Seventy mice were acclimated under constant environmental conditions for several days. They were then assigned at hazard in groups of 10, injected with the chosen dose of choline chloride, and placed singly in plastic cages. The number dead by 1 hr after injection was recorded. Values for LD50 and their confidence limits were calculated by a reiterative regression-line approximation program.

(ii) Cumulative toxicity. Fifty mice were used to investiage the possibility that choline toxicity might be cumulative. Choline chloride,

160 mg kg^{-1} was administered at three successive 12 hr intervals (11 A.M., da 1; 11 P.M., da 1; 11 A.M. da 2). A second group was injected with saline at 11 A.M. and then with choline chloride 160 mg kg^{-1} at 11 P.M. and again at 11 A.M. on the next day. The number dead 1 hr after injection was recorded.

(f) "Free" and "bound" ACh in morphine-dependent rats. The estimation of "free" and "bound" ACh levels found in rat brain during morphine dependency and withdrawal was carried out by a method modified from Crossland and Slater (1968). Three groups of 6 animals - saline controls, morphine dependent, and 24 hr morphine withdrawal - were sacrificed by decapitation. The brain from each animal was immediately removed, weighed and immersed in a cold ($4^{o}C$) saline solution containing physostigmine (15 µg ml^{-1}) to prevent ACh breakdown, and cupric chloride (17 µg ml^{-1}) to prevent ACh synthesis, in a ratio of 5.0 ml gm^{-1} of brain tissue. The contents were homogenized in the cold ($5^{o}C$) at approximately 1500 rev min^{-1} for 3 min and then immediately centrifuged at 2300x g for 30 min. The supernatant was adjusted to pH 4.0 with 0.5 N HCl and allowed to settle for 30 min at which time the clear solution was decanted off the remaining precipitate. This clear solution contained the "free" ACh.

The residue left after extraction of "free" ACh was then gently ground for 5 min in an acid-ethanol solution using a tightly fitting pestle and centrifuge tube. The solution consisted of 2 ml of glacial acetic acid in 1L of ethanol, this was added to the residue in a ratio of 5 ml for each gm of original brain tissue. The mixture was centrifuged at 37,000x g after standing for 20 min. The supernatant was removed and the second residue washed twice with another acid-ethanol solution containing 15 ml of glacial acetic acid in 1L of ethanol. This

was added to the residue in a ratio of 2.5 ml for each gm of original brain tissue. Supernatant and washings were combined and evaporated under vacuum. The powder was reconstituted with a saline-physostigmine solution and adjusted to pH 4.0. This constituted the "bound" fraction. Aliquots were then assayed immediately on the clam heart (Mercenaria mercenaria; obtained from Pacific Biomarine Supply) bioassay (Florey, 1967; Tower and McEachern, 1948) using a "bracketing" technique between known concentrations of ACh, or were frozen and stored for future estimation.

(B) TREMOR STUDIES

It was felt, in preparing this dissertation, that a critical evaluation of previously accepted methods for measuring tremor would be essential for proper evaluation of and comparison with the methods developed and used in the present study. Therefore the pertinent historical background of tremor measurement has been presented as part of the "METHODS" section of this thesis. Its incorporation into the "INTRODUCTION" section was felt not to be justified, since that section is meant to deal solely with the central biological hypothesis of this work.

1. Background of methods used by others.

(a) Classification of tremor. Tremor is a relatively common response to a variety of substances. Although the term is used frequently,

it has a spectrum of connotations ranging from the violent muscular contractions seen in epileptic convulsions to the fine and often barely measurable movements of normal human extremities. The criteria for classification of movements as tremor vary amongst authors but in general they include its involuntary nature, its rhythmic, regular, and oscillatory pattern, and its apparently purposeless motivation. Brimblecomb and Pinder (1972) define tremor as "a series of spontaneous, involuntary, purposeless, oscillatory motions involving any part of the body that is moved by skeletal muscle. This trembling or shaking movement can be regular or irregular, continuous or sporadic, fine or gross and can be present at rest or with activity".

Brumlik and Yap (1970) have surveyed the various classifications of tremor and have devised a new classification based on the degree of postural innervation that exaggerates the tremor. They have divided tremor into two main groups: Normal (Physiological) and Abnormal (Pathological). Each is further subdivided into either rest, postural or intentional tremor. "Rest" refers to tremor in relaxed voluntary muscles; "postural" indicates that the part involved is held against gravitational forces (as in an outstretched hand) and is an isometric contraction involving no goal; "intentional" tremor refers to that seen when the part is moving towards a goal and is an isotonic contraction.

Thus, all "normal" tremor is characterized by waves of fine amplitude at a frequency of 8-12 hr. Abnormal tremor is found in various disease states: "rest" tremor is typical in Parkinson's disease, "abnormal postural" occurs in delirium tremens, thyrotoxicosis and shivering, and "abnormal intentional" tremor in Wilson's disease and multiple sclerosis.

In addition to the above types of tremor which have a purely physio-

logical or pathophysiological origin, there are tremor states that may be induced by various drugs. Such tremorigenic agents have a diversity of structure and it is therefore helpful to classify them into smaller groups when studying mechanisms of actions. Stern (1969) classified several drugs on the basis of the type of tremor they produced and the ability of other drugs to prevent or reverse the tremors. It is interesting to note that Brumlik and Means (1969) have shown that the drug tremorine, which was initially used to simulate Parkinson-type tremor, does not in fact produce tremor in the dog, at a frequency similar to that seen in that disease.

Consequently, it may be more meaningful to classify drugs according to the particular biogenic amines which appear to be involved in the production of the tremor, as was done by Brimblecombe and Pinder (1972). Drugs are then classified as (1) aminergic (tremor is mediated by catecholamines or 5-hydroxytryptamine (5-HT)), (2) cholinergic (ACh is the mediator) and (3) dual or multiple (with combinations of (1) and (2) and possibly others such as histamine).

In this study, drugs which generally fall into the aminergic and cholinergic classes were utilized. Harmine was used as a representative of the aminergic class while tremorine and oxotremorine (XTR) were employed to study cholinergically-mediated tremor. Hence a review of the literature concerning these agents is appropriate here.

(b) Drug-induced tremor.

(i) Harmala alkaloids. These compounds have been known for their marked effects on the CNS for nearly a century (Friedman and Everett, 1964). Within this class of drugs there are many compounds which differ

only slightly in their pharmacological actions.

Harmine is an alkaloid of the seeds of Peganum harmala, a plant which grows in the steppes and wild places of the Mediterranean and East Indian Belt. It has been used for centuries for a variety of conditions, but in particular, as an anthelminic. The harmala alkaloids produce a variety of central and peripheral actions including visual hallucinations, respiratory stimulation, cardiac depression, skeletal muscle contraction, tremors and clonic convulsions (Sollman, 1957).

The actions of the alkaloids were first investigated by Tappeiner in 1895. He described the central effects of harmaline as well as those on the circulation, and estimated roughly its lethality. In mammals, harmine in large doses causes tremors and convulsions, the latter varying in different species (Gunn, 1935). Lethal doses produce respiratory paralysis and hypothermia. It also provokes a fall in blood pressure and cardiac arrest during diastole. The alkaloids produce muscle relaxation except in the uterus, where they increase strength of contraction.

Although there have been considerable studies on harmala alkaloid-induced tremor, several have been concerned only with the pharmakokinetics of the various compounds and not with their mechanism of action (Zetler et al., 1972; Singbartl et al., 1973; Zetler et al., 1974).

Hara and Kawamori (1954) studied the effects of harmine on the extrapyramidal system. Despite its use as a treatment of rigidity in postencephalitic Parkinsonism (Astley Cooper and Gunne, 1931), Hara and Kawamori refer to it as an extrapyramidal poison. They found that methamphetamine enhanced tremor produced by harmine, but that nicotine and eserine did not. Moreover, bulbocapnine, a cataleptogenic agent similar in structure to apomorphine but lacking its emetic effect (Franz, 1975),

markedly reduced harmine tremor. Finally, ablation of the cerebral cortex or striatum abolished the tremor, suggesting an extrapyramidal origin of this tremor.

Zetler (1957) conducted an extensive investigation of the antagonism of harmine-induced tremor because he felt that this relatively long-lasting, coarse tremor was a good test-situation for the evaluation of antiParkinson agents. He found that the phenothiazine tranquilizers were more potent antagonists than were the anti Parkinson agents and that their sedative and cholinergic blocking activity did not correlate with this antagonism. A combination of adrenolytic, sedative and antitryptaminergic actions provided the greatest antagonism, with LSD, serotonin and chlorpromazine the most potent of the 41 agents tested. There was only a small difference between the blocking actions of atropine and scopolamine. Yen and Day (1965) noted that most classes of drugs tended to reduce harmine tremor but that chlorpromazine and lysergic acid were the most effective of those used. These results agree closely with those of Zetler (1957). Also, atropine did not antagonize this tremor.

Agarwal and Bose (1967) investigated the role of brain catecholamines in tremorine- and harmine-induced tremor. They found that one beta-adrenergic blocker, d-INPEA, did not modify harmaline tremor, whereas propranolol, another beta-blocker known to also have CNS effects, did reduce tremor. They suggested that it may be the CNS action which is responsible for the anti-tremor effect. Furthermore, depletion of brain NE and dopamine by AMT did not alter tremor, wherease increases or decreases in these catecholamines with concomitant changes in serotonin produced by pargyline and reserpine, respectively, both enhanced harmaline tremor. The latter results are paradoxical and difficult to explain.

Bowman and Osuide (1968) studied the effects of a large number of drugs on tremorine and harmine in chicks. Amphetamine, dopamine, dopa and reserpine (24 hr previously) increased harmine tremor, whereas 5-HT, 5-hydroxytryptophan (5-HTP), and chlorpromazine reduced the tremor. Drugs acting on the cholinergic system (hemicholinium, physostigmine and deanol) were without effect.

Cox and Potkonjak (1971) support the non-cholinergic mediation of harmine tremor. Moreover, they found (and later repeated by Coates and Cox, 1972) that its inhibition of monamine oxidase (Udenfriend et al., 1958) cannot account for the tremor; mebanazine, another inhibitor of brain monoamine oxidase did not produce tremor on its own, nor did it affect harmine tremor.

In a series of studies Kelly and Naylor (1974, 1975, Costall et al., 1976) attempted to systematically examine the neurotransmitter basis for, as well as the central sites of, harmine tremor in the rat. Anti-cholinergic drugs failed to modify tremor; dopamine agonists reduced tremor and monoamine depleting agents enhanced tremor. Their results on the modification of tremor by 5-HT are difficult to assess since 5-HT changed the nature as well as the magnitude of the tremor, and the latter was increased or decreased as a function of dose. These results and the observation that 5-HT and harmine are structurally similar, led to their conclusion that 5-HT is probably involved, in some way, in the tremor response. They also implicated a dopaminergic mechanism and suggested the possibility of a central dopamine-5-HT interaction (1974). Using the brain lesion technique (1975), they found that lesions of the caudate-putamen or substantia nigra failed to modify tremor, as well as its antagonism by dopaminergic agonists. Bilateral lesions of the globus

pallidus, known to be rich in dopamine (Broch and Marsden, 1972), however, resulted in reduction of tremor intensity as well as a decrease in the effectiveness of dopaminergic agonists in their ability to block tremor. It is difficult to reconcile these results with those of Cox and Potkonjak (1971) and Larochelle et al., (1971) who have shown that stereotaxic injections of harmine in the caudate-putamen and substantia nigra produce tremor. Moreover, Kelly and Naylor (1975) point out that much of the input to the neostriatum is from the globus pallidus, and so they are indirectly modifying activity of the former structure by their lesions in the latter.

Finally, Costall et al. (1976), this time lesioning midbrain raphe nuclei, concluded that harmine normally induces tremor by enhancing 5-HT function. They again observed that l-DOPA and apomorphine were able to reduce tremor and that in lesioned animals this effect was reduced. Their discussion emphasizes the interaction of 5-HT and dopamine, centrally, in harmine-induced tremor.

It should be apparent from the foregoing discussion that the precise mechanism for harmine-induced tremor is not yet known. Nevertheless, the great majority of papers have implicated a 5-HT or catecholamine basis for this tremor and have discounted the possible role of the cholinergic system as a mediator of the harmine response. Hence, for the purpose of the studies presented here, it may be assumed that harmine tremor is non-cholinergic in nature.

(ii) Tremorine and Oxotremorine. Tremor-producing agents were found with an incidence of about one in a thousand in a general screening program (Everett et al., 1956a, 1956b). The ability of an agent to produce tremor as well as obvious cholinergic signs was even rarer. Tremorine, 1,4-dipyrrolidino-2-butyne, was such a compound. In doses of 5 to 20 mg

mg kg^{-1}, given intraperitoneally, animals developed a syndrome composed of sustained tremor of the head and extremities, parasympathetic signs of diarrhea and profuse salivation, analgesia, anergia, and hypothermia. The authors reported that atropine was very efficacious in preventing the tremor and that peripheral cholinergic blocking agents such as methantheline, could prevent the salivation and diarrhea, but were ineffective against the anergia and hypothermia. Moreover, central depressants such as the barbiturates, alcohol, anticonvulsants and analgesics were ineffective. They suggested that this compound could be used to characterize both central and peripheral cholinergic blocking drugs.

After the initial studies by Everett et al. (1956a), tremorine became popular as a tool for delineating central and peripheral cholinergic actions of various drugs. It has been used extensively as a tremor-producing agent to simulate Parkinson's disease and it is this latter effect which has prompted many investigations of its mode of action on the extrapyramidal motor system. In the present study tremorine and its active metabolite, oxotremorine have been used as a means to monitor cholinergic activity and sensitivity during chronic morphine administration.

Kocsis and Welch (1960) found that incubation of tremorine with liver slices resulted in the accumulation of a compound with intense pharmacological activity. Unlike tremorine, this substance produced immediate tremor upon injection into mice and was active in very small doses. The metabolite was named oxotremorine (XTR; Cho et al., 1961, 1964). In a review of its peripheral actions (Cho et al., 1962), the authors indicated that XTR produced a fall in arterial blood pressure,

a negative chronotropic and inotropic effect in vitro, intestinal smooth muscle stimulation, and profuse salivary secretion. All these effects could be prevented or abolished by atropine.

Chen (1958) determined that tremorine was analgesic in doses smaller than those required to induce tremor. Using the Haffner tail-clip analgesia test, she found that hyoscine and atropine antagonized the tremorine analgesia and that nalorphine and phenobarbital were ineffective. She concluded that tremorine's mode of action was different from that of morphine and that this test could be used to screen potential antiParkinsonism agents.

Pepeu (1963) failed to demonstrate a causal relationship between a rise in brain ACh and tremorine-induced tremor because there was also an associated elevation in brain histamine and a depression of brain NE. Friedman et al. (1963) found that there was a significant decrease in brain stem NE after tremorine as well as a rise in 5-HT. However, after bilateral adrenalectomy, NE levels were even lower but 5-HT was not changed, and tremor was less pronounced. These latter results are not in agreement with Bernheimer et al. (1973) who studied brains from patients with Parkinson's disease and found depressed levels of 5-HT and NE. Nevertheless, Friedman et al. (1963) postulated a peripheral as well as a central involvement in tremorine tremor and possibly in Parkinsonism; they suggested that the peripheral effects may modulate those in the CNS by a feedback mechanism.

Leslie and Maxwell (1964) investigated the differential efficacy of several phenothiazine derivatives in blocking tremorine- and XTR-induced tremor. They found that, whereas the phenothiazines were able

to prevent tremorine tremor, they were ineffective against the XTR effect. SKF-525A, a potent inhibitor of liver metabolism, acted similarly. Since XTR is the active metabolite of tremorine the investigators arrived at the obvious conclusion that the phenothiazines may be acting by inhibiting the activation of tremorine.

Patten et al. (1964), questioned the extrapyramidal origin of the tremor produced by tremorine, as well as its use in appraising potential antiParkinson drugs. They indicated that poikilothermic animals did not develop tremor nor parasympathetic manifestations, whereas homeothermic species showed both. Also, tremor could be dissociated from the parasympathetic effects by tranquilization, either by handling, or by reserpine administration, with the tremor disappearing. This was in contrast to the situation in Parkinson's disease where it was the tremor which was resistant to treatment. They concluded that their results supported the data of others which indicate that tremorine was not acting at the level of the basal ganglia but was probably rather at the level of the hypothalamus.

Spencer (1965) determined that the hypothermic action of tremorine could be used more reliably than tremor as an indicator for potential antiParkinson drugs. He found that centrally-acting drugs could antagonize the hypothermia and tremor but that the two responses were separate entities with different sensitivities. Quaternary anticholinergic drugs such as propantheline bromide and atropine methylnitrate, which do not gain access to the CNS, were unable to prevent either the hypothermia or the tremor induced by tremorine. They did reverse the peripheral effects of tremorine indicating that the former responses were

indeed central in origin. Also, by comparing the relative sensitivities of these two effects to various agents, Spencer was able to show that drugs with either sympathetic or anticholinergic effect could be differentiated on the basis of their ability to reverse the hypothermia and tremor induced by tremorine. Cholinergic blockers were equally effective against both effects whereas the sympathomimetic drugs were more effective against the hypothermia than against the tremor.

Spencer has recommended that the antihypothermic effect should be used to evaluate potential antiParkinson agents in the tremorine test procedures. He argues that, while many agents have been reported as effective in blocking or reversing tremor, only those drugs with anticholinergic or sympathetic activity are useful in reversing the hypothermia.

Spencer (1966) was concerned with the descrepancy amongst investigators as to whether the thymoleptics (i.e. tricyclic anti-depressants) were preventing tremorine tremor by a true pharmacological action or by inhibiting its activation. Leslie and Maxwell (1964) had reported the latter mechanism; Theobold (1964) reported that it was an anticholinergic mechanism, and Spencer (1965), that it was a sympathomimetic effect. In the 1965 paper, Spencer confirmed his earlier results and found that the thymoleptics could abolish established tremor. This indicated that, in addition to an inhibitory effect on liver metabolism, these agents also had a direct effect at the site of tremorine action in the mouse. It appears, therefore, that the rat and mouse have enzyme systems which differ from each other.

Agarwal and Bose (1967) investigated the role of brain catecholamines in drug-induced tremor. They found that one beta-adrenergic

blocker, d-INPEA, had no effect on the tremor produced by tremorine or harmaline. In contrast, they found propranolol effective against tremorine but not against harmaline. They concluded that propranolol was probably acting in a way not dependent on beta receptors. Also, depletion of NE and dopamine by alpha-methylparatyrosine (AMT) did not modify the tremor produced by these drugs. In contrast, depletion of brain catecholamines and 5-HT by reserpine, or their elevation by pargyline, diminished tremorine tremor and enhanced harmaline tremor. It appears from those results that there is some optimal level of relative neurotransmitter activity in the brain, and that any change from the normal range, (regardless of its direction) disrupts a tremor homeostasis mechanism. The authors concluded that catecholamines probably played a permissive role in the production of drug-induced tremor, and were not by themselves directly involved in the tremor response.

Slater and Rogers (1968) investigated the effects of triethylcholine and hemicholinium on the increases of brain ACh induced by tremorine, harmine and eserine. Triethylcholine and hemicholinium compete with choline for access to the intracellular sites at which synthesis of ACh takes place, and this effect can be prevented by simultaneous administration of choline. Both tremorine and eserine produced increases in "free" and "bound" ACh which were prevented by prior administration of triethylcholine and hemicholinium; these latter two also prevented the tremor. Harmine tremor was not affected. They postulated that the excess of "free" ACh could be responsible for the tremorigenic activity of XTR. In addition they presented the notion that XTR must have a direct effect as well, because eserine, which causes a similar increase in

"free" and "bound" ACh, does not produce as marked a tremor response. That triethylcholine and hemicholinium were not behaving in an atropine-like manner was evidenced by their failure to prevent tremor when accompanied by choline administration. Importantly, the results indicated that harmine was not acting via a cholinergic route.

Bowman and Osuide (1968) confirmed the results of others in showing that tremorine-induced tremor has a large cholinergic component. Using chicks, they found that the tremor response was depressed by atropine, hyoscine, propantheline, orphenadrine, chlorpromazine, hemicholinium and others. An interesting point was that a quaternary derivative, propantheline, was effective in a presumably centrally-occurring phenomenon. This was explained readily by the fact that the chick has an undeveloped blood-brain-barrier and therfore allows free access to this compound. Although tremor produced by tremorine did not appear to be directly attributable to the changes in the level of catecholamines, it was nevertheless affected by changes in their levels. This may have been due to the known facilitation of cholinergic synaptic transmission by adrenaline (Birks and MacIntosh, 1961).

There are considerable species differences in the hypothermic and tremorigenic actions of tremorine and XTR. Hammer et al. (1968a,1968b), proposed that these differences were attributed to differences in the rates of metabolism of these compounds. The effects of these drugs are short lasting in the rat but maintained in the mouse. They found that these compounds were metabolized quickly in the rat but slowly in the mouse and that brain levels were nearly 20 times higher in the mouse than in the rat. By inhibiting the metabolism of XTR with SKF-525A, they obtained elevated brain levels and consequently a proportional degree

of hypothermia in the rat. In contrast, this inhibitor had little effect in the mouse, suggesting that there are differences in the relative importance of various metabolic pathways in rats and mice. Even after pretreatment with phenobarbital, a well-known inducer of hydroxylating enzymes, the half-life of XTR was over 3 hr in the mouse. Hence, qualitative and quantitative species differences in the pharmacological actions of the agents could well be explained by the metabolic limitations in these species.

The profound hypothermia induced by these tremorigens contributes to their prolonged effect, because of a diminished metabolic rate. Hammer et al. (1968a, 1968b), suggested that drugs which counteract the hypothermia also enhance the metabolism; they therefore increase the clearance of XTR from the brain, thereby decreasing the intensity and duration of the tremor.

Cox and Potkonjak (1969a) reported that atropine inhibits XTR tremor without preventing the increase in brain ACh which results from XTR administration. It appeared, therefore, that atropine was effective by some action on central muscarinic receptors. If this were the case, atropine would inhibit XTR tremor whether the latter acted directly, or by an ACh-releasing mechanism. They also concomitantly administered dyflos, a cholinesterase inhibitor, to determine whether the increased ACh would potentiate the tremor. It failed to do so. It appeared, therefore, that this tremor was produced by a direct receptor interaction.

In a further study, Cox and Potkonjak (1969b) ran a series of studies on the time relationship between tremorine- and XTR-induced tremor in the rat and the corresponding changes in brain ACh.

Tremorine, in a dose of 20 mg kg^{-1} caused tremor and an increase in brain ACh within 5 min. However, whereas the tremor reached a peak in the first 20 min, the brain ACh did not reach its maximum until 30 min. In contrast, XTR in a dose of 0.5 mg kg^{-1} produced tremor in 30 sec but there was no measurable change in brain ACh 5 min after the injection. Higher doses did cause elevations in brain ACh. These data did not indicate a causal relationship between tremor and brain ACh but did not eliminate the possibility that changes in ACh levels in discrete areas of the brain were occurring which could not be detected on a whole brain measurement. In support of this is the ability of XTR to induce tremor when administered stereotaxically in the substantia nigra (Cox and Potkonjak, 1969c).

Sharma (1970) reported that tremorine tremor could be antagonized by beta receptor antagonists. He found that dl-propranolol and dl-INPEA were effective whereas their dextrorotatory isomers were ineffective. None of these agents prevented tremorine-induced lacrimation or diarrhea, but hyoscine did. This suggested that the anti-tremor activity of the beta antagonists was not due to an atropine-like action. Sharma's paper is supported by many clinical findings.

In Parkinsonism the dopamine content of the substantia nigra is diminished (Hornykiewicz, 1966) and monoamineoxidase inhibitors reduce Parkinsonian tremors (Barbeau, 1960). Adrenaline and isoprenaline, but not NE,potentiate Parkinsonian tremors, these were reversed by the beta-adrenergic blocker propranolol but not by the alpha-blocker phentolamine (ibid). Despite these several important observations, there have been relatively few reports on the involvement of catecholamines in XTR-induced tremors.

Although a direct-acting central muscarinic effect of XTR was reported by Cox and Potkonjak (1970), they also found a wide range of non-cholinergic agents which modified the tremor response. They found that reserpine, AMT and diethyldithiocarbamic acid, drugs which lower tissue NE, were capable of inhibiting the tremor without preventing the increase in brain ACh. Phenoxybenzamine and propranolol also inhibited tremor but it appeared that 5-HT did not. These results implicated a role of NE in XTR tremor, but some of their results were in conflict with those of others. They have criticized others for the low doses of PCPA used and did not consider that their own doses (i.e. 300 mg kg^{-1}) may have been toxic. Moreover, they did not determine whether phenoxybenzamine was inhibiting via an atropine-like action.

Their measurements of brain ACh indicated that there was no relationship between the modification of the tremor response and the changes in brain ACh. This did not exclude the possibility that XTR was acting directly and therefore any coincident changes in brain NE would cause compensatory changes in brain ACh levels and appear haphazard.

Studies that induce depletion of specific transmitters have always been open to criticism with respect to levels of depletion required, to the degree of safety factor which exists at a given site, to compensatory mechanisms which come into play and disrupt the "normal" state and to subtle toxicities. A more physiological approach would be to measure changes in neurotransmitter levels which result from drug-induced changes. It would be even more useful to measure synthesis and removal ("turnover") of neurotransmitter substances in the CNS. Rogers and Slater (1971) administered a variety of agents and measured the concom-

itant changes in brain monamines. They found that physostigmine and atropine both increased brain 5-HT, indicating that 5-HT levels cannot be used as a reliable indication solely of monoamine activity. Tremorine caused a decrease in brain NE, but the time of peak decrease occurred during a diminishing tremor response. Atropine prevented the tremor and the depletion of NE but hemicholinium and triethylcholine, which prevented the tremor, had no effect on brain NE. This discrepancy could be explained by the fact that the tremorine tremor response may be due to a combination of direct actions and an increase in free ACh, and therefore, hemicholinium can inhibit some of the tremor by preventing this increase in free ACh. Atropine prevents tremor via both mechanisms. If it is cholinergic receptor agonism which causes release of NE then atropine would be effective. Hemicholinium would be ineffective because XTR could still activate the receptors.

Menon et al. (1971) found that tremorine, even in toxic doses, did not alter brain histamine levels.

Cox and Hecker (1971), using the isolated guinea-pig ileum preparation, found similar log dose-response curves to XTR and ACh; both drugs were competitively antagonized by atropine. There was no evidence of ACh releasing actions and they concluded that it acted directly on muscarinic receptors.

Leslie et al. (1971) found four beta-blockers that were more efficacious against tremorine than against XTR, indicating that there may have been some interference with the metabolic activation of tremorine. Practolol was the only agent which did not produce hypothermia and it was the least effective against tremorine, suggesting that the antagonism of the other agents was due to the slowing of metabolism produced by the

hypothermia. On the other hand, propranolol, the most potent beta-adrenergic blocker among those tested was also the most effective tremor blocker. Hence, the interpretation of these results is questionable.

Choi et al. (1973) found that XTR inhibited the turnover rate of ACh to the same degree in all brain regions. Szerb and Somogyi (1973), studying cerebral cortical slices, found that XTR inhibited ACh release and that this was reversed by incubation with atropine. This inhibition of release can account for the increased ACh content reported in the foregoing. Sethy and Van Woert (1973a, 1973b) found a correlation between the elevated ACh and tremor induced by XTR and physostigmine. Antimuscarinic drugs prevented these changes. L-dopa did not alter the effect of XTR when given in a single dose.

Cox and Tha (1973) used three tests to determine whether XTR was acting directly or via an ACh mechanism. Toxicity, esophageal temperature, and tremor were the indices measured because they allowed the administration of a wide range of doses, and therefore, would detect any interactions with the anticholinesterases, physostigmine and dyflos. They found a lack of potentiation over a 300-fold increase in dose and therefore concluded that the peripheral, as well as the central effects, were due to a direct action of this drug.

Slater (1974) investigated the effects of 6-OHDA on the hypothermia, analgesia and tremor produced by tremorine and XTR. He found that tremor and hypothermia were not affected by the resulting NE depletion in the rat but the administration of pargyline and 6-OHDA, which depleted dopamine as well as NE, did prevent the hypothermia. In the mouse, XTR failed to increase the response time in the hotplate test after intraventricular 6-OHDA. These results suggest that mono-

amines are not involved in tremor but that dopamine may play a role in the phenomena of hypothermia and nociception. There remained the possibility, however, that there was insufficient depletion of NE with the 6-OHDA administration.

Nose and Takemoto (1974) found that XTR and several cholinomimetics such as arecoline, nicotine and physostigmine, all increased the homovanillic acid concentration in the rat striatum. The increase caused by XTR was prevented by atropine but not by atropine methylbromide, mecamylamine (a ganglionic nicotinic blocker with central effects), nor by hemicholinium. These results indicated that the action was at a central level and that it was a direct receptor effect of the muscarinic type. The increase in dopamine metabolism suggested that there was a dopaminergic-cholinergic interaction regulating the rate of dopamine formation.

A similar study was done by Anden (1974) using rabbits. He found that there were increases in homovanillic acid in both the striatum and limbic system after XTR and physostigmine. After blockade of peripheral and central muscarinic receptors by trihexphenidyl, the changes in the striatum were completely inhibited, whereas there was only a slight inhibition in the limbic system. This differential effect suggested that either there were different ACh receptors in these two regions or that the arrangement of the neuronal interaction was different. It appears plausible that the limbic system may lack a dopaminergic feedback, thus being insensitive to a cholinergic block.

Ganguly (1976) investigated the site of action of propranolol's ability to block the tremor produced by XTR, 250 µg/kg intraperitoneally,

in mice. He found a dose-related inhibition in the whole animal and using isolated tissues, attempted to ascertain where and how propranolol was acting. Propranolol alone did not reduce the twitch height of indirectly stimulated rat diaphragm, but did partially prevent the block induced by XTR. In addition, propranolol did not decrease the response to exogenous ACh in the frog rectus abdominis, did not alter the vasodepressor response to XTR in the rat and did not alter the spasmogenic response in guinea-pig isolated ileum. He concluded from these experiments that propranolol may act presynaptically at the neuromuscular junction and thereby decrease tremor. He failed, however, to rule out a central action of XTR.

(c) Methods.

(i) Introduction. In studies on tremorigenic substances it is necessary to have accurate and reproducible methods for measuring tremor. In some experimental instances, determination of the presence or absence of tremors may suffice, whereas in other studies the quantification of severity, and analysis of waveform or frequency, may be required. Basically, therefore, the methods used fall into two classses: observational or instrumental.

(ii) Observational. These methods involve the observation of animals for the presence or absence of tremors. Animals are injected and then observed for various durations which are determined by the particular drug being administered. Using this method, Brimblecombe et al. (1970) were able to determine values for ED50 and ED95 of XTR. It is apparent that this method is entirely subjective and that results may vary from observer to observer and even from experiment to experiment.

The observational method has been used also to evaluate the severity of tremor, by assigning arbitrary scores to various tremor states (Cho and Jenden, 1964). In their study, a score of 3 was given for continuous incapacitating tremor, a score of 2 for intermittent spontaneous tremor and a score of 1 for tremor which appeared only on restraint. This method was also used by Sethy (1973) but, to increase reliability, two people made independent assessments of tremor severity.

The various observational methods are quite satisfactory for comparative studies in a standard situation, such as in pharmacological screening, where economic factors dictate the duration and complexity of the experiments. They are, however, of little value in the analysis of small changes of tremor, both in degree and type, unless one uses an expanded scale for rating, and is confident that the assigned values are reproducible between observers.

(iii) Instrumental. Many instrumental methods for recording tremor in an objective manner have been described in the literature and are reviewed in the text by Brimblecombe and Pinder (1972). Randall and Metzger (1963) maintain that body tremor may be measured quantitatively using methods which measure force, displacement, velocity or acceleration. Various optical methods, using light beams, and mirrors and lenses, have been used to measure displacement (Brimblecombe and Pinder, 1972). These do not add to the inertia of the body and are sufficiently sensitive to record fine tremors. However, they may be too sensitive to measure gross body movement and also, they often must be used in the dark.

Strain gauges, capable of measuring force or velocity, have been used in a Wheatstone bridge system, arranged in such a manner that the

bridge balance is upset by forces on the strain gauge. In this case inertia is added to the moving part. The record is not linear but is a composite wave form of movement in many planes (Slater and Rogers, 1968). Electromyography, employing electrodes which record action potentials that accompany the mechanical motion of tremor, has also been used. This may be uncomfortable for the animal, particularly if the electrodes are placed intramuscularly. The record itself represents bursts of action potentials which may not necessarily correspond to the tremor and may indeed confuse gross body activity with tremor (Wachs and Boshes, 1961).

An electromagnetic device for recording tremors in mice was described by Moore et al. (1957). Small permanent magnets were implanted subcutaneously in the neck region and the animals were placed in a glass tube wound with a coil. The frequency of the voltage induced in this coil was directly proportional to the rate of movement of the mice, while its amplitude was related to the velocity of the motion. As Ahmed and Taylor (1959) point out, animals may die from sepsis even if precautions such as sterilizing magnets are taken. Dill et al. (1968) have used the identical method using small magnets taped to a rat's forelimb instead of surgical implantation and have thereby eliminated the time and trauma involved in such preliminary procedures. Nevertheless, the tremor measured may not be a sensitive measure of total body tremor.

Most workers, however, have placed their animals in small boxes and then recorded the movements of the box by use of a simple mechano-electric transducer, as a phonograph pickup head. The movements of the animal are thus converted into electrical energy which may then be fed into either an oscilloscope for photographic purposes, or into a pen

recorder for more continuous recording. Both qualitative and quantitative measurements can be obtained in this fashion since the tremor signal can be fed into an integrator after its initial processing (Cox and Potkonjak, 1969; Ahmed and Taylor, (1959). Analysis of tremor frequency has been attempted by many investigators but conclusions often appear invalid as the resonant frequency of the box is usually unknown or has not been specified. The influence of this parameter on the apparent frequency of tremor is also unknown in those experiments. Box resonance may actually appear to be a tremor signal when the latter is similar to any significant component of the body tremor; this will obviously confound the results.

In summary, the ideal method of measurement would be one in which no surgical preparation was required and one in which an accurate, easily quantified record is obtained in which there is no contribution by extraneous variables which may arise as a result of instrumental limitations.

2. Methods used for measurement of tremor in this study

(a) Apparatus. The device for detection and measurement of fine body tremor was a simple plastic box fitted with a hinged cover and suspended at one corner by a spring-steel blade (Fig. 1). A piezoelectric transducer element, removed from a standard phonograph cartridge, was bonded to the blade. The box was suspended by a clamp on the spring blade and the entire assembly mounted on a slotted steel table of 25 kg mass. A damping pad (Fig. 5) of polystyrene foam was fitted between the box bottom and slotted steel table. Compression of this pad was adjusted to obtain just less than critical damping. The table itself was vibration damped by four shock absorbers: firm sponge-rubber playground balls, compressed to one-half their 6 cm diameter. The slotted table sat on a

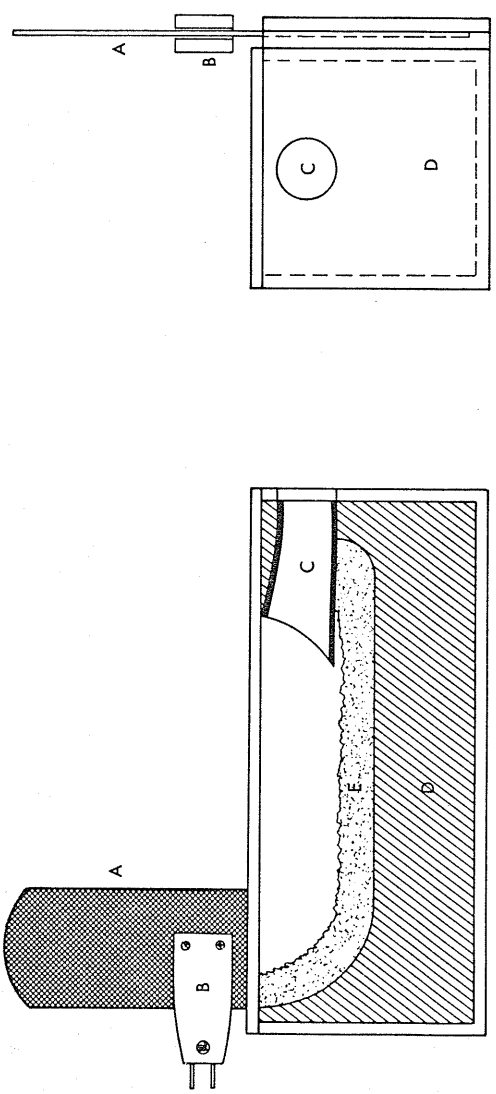

Fig. 1. Tremor box; detail.

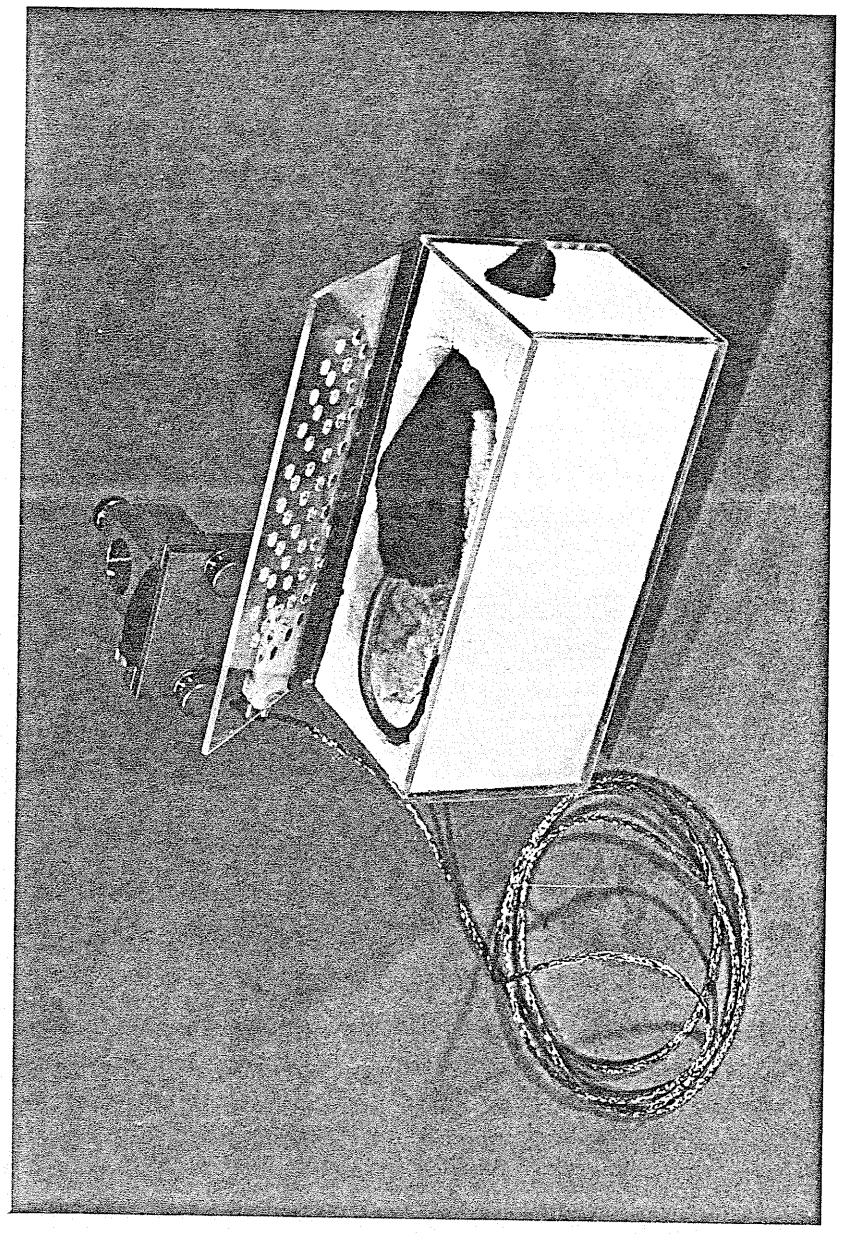

Fig. 2. Tremor box, photograph.

standard laboratory bench which stood on the reinforced-concrete floor of the laboratory. The system was unresponsive to all normally-encountered building vibrations, to normal laboratory traffic and to all but the most exaggerated efforts to induce artefactual vibrations, such as hammering on the bench or jumping vigourously on the floor nearby.

The mouse being tested was placed in the plastic tremor box (Fig. 2) and held in place there snugly, but without undue restraint, by a contoured foam plastic bed insert. A shaped snout piece induced the animal to poke the head forward and thereby breathe quietly and comfortably. At random intervals almost all animals within the tremor box poked their heads forward into the snout piece and scraped at the foam insert with their claws. This activity was not sufficiently frequent to interfere with the measurement of fine body tremor. None of the animals ever vocalized indications of distress or fear during handling, injection, placement in the box, or during restraint within it. Moreover, those animals which were tested more than once in these and other studies in the laboratory, appeared increasingly to tolerate placement and restraint within the box at each successive trial.

Vibrations of the box due to fine body tremor were transmitted to the spring blade-transducer system and an electrical output, corresponding in amplitude to the velocity and frequency of the tremor, was obtained from the transducer element. This electrical signal was amplified, rectified and averaged with a capacitance-resistance filter network having a 1-second time constant (Figs. 3,4). A pen recording of this averaged activity provided a graphic record of tremor activity (Fig. 8). This record was not affected by the frequency-response limitations of the ink-recording galvanometer (6 db down at 30 hz) because the signal produced

by the prior averaging and rectifying (Fig. 4) was well within its effective range.

In order to quantitate the "response" characteristics of the tremor apparatus, several parameters were examined, using a loud-speaker (Goodman, 8', 15-watt)-driver arrangement as shown in Fig. 5. Frequency and driving power could be varied independently with this system and the corresponding outputs from the transducer monitored. Some of the salient characteristics of the tremor apparatus are shown in Fig. 6 and 7. Fig. 6 shows that the system output is linear over a range of vibration energy which results in transducer output signals of up to 1.6 volts RMS (root mean square); this range was well within that produced by the tremor activity observed in these studies. Moreover, this linearity was not disturbed by the presence of a mouse in the box (Fig. 6). A constant driving frequency of 50 hz was used here to check linearity because it was close to the upper limit of the frequency components of the tremor signal. Linearity was fully maintained when a frequency of 100 hz was used.

The frequency response of the box itself is shown in Fig. 7. The peak response occurred at a frequency of 100 hz; this was well above the tremor frequencies encountered with the tremorigenic agents used in these experiments as well as in those used by other investigators. This was an important point since the resonant frequency of the container conceivably could affect the tremor signal and lead to confounding results. This precaution, if taken by other investigators, has not been noted in most publications on tremor and indeed, may have led to the conclusion that the predominant frequency components of tremorine and XTR tremor lie between 14-18 hz. We have found, using various frequency filters, that although 18 hz was a major component of this tremor, there

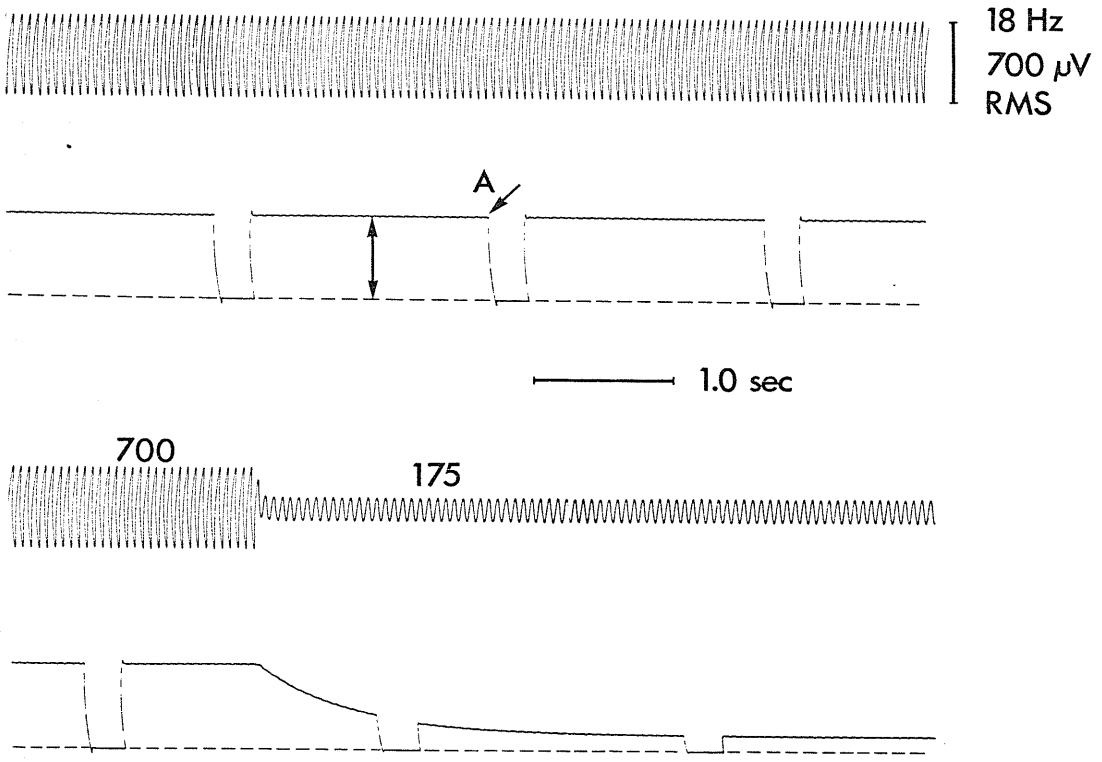

Fig. 3. RMS Tremor Energy Detector; measurement of signal amplitude and response-time characteristics. Height of vertical arrow is proportional to RMS amplitude of tremor signal. At "A" the averaged tremor signal is interrupted for comparison with zero-signal baseline (dotted). In experiments, interruptions occurred every ten seconds. Bottom trace shows system response to abrupt change of input signal amplitude (from 700 μV to 175 μV).

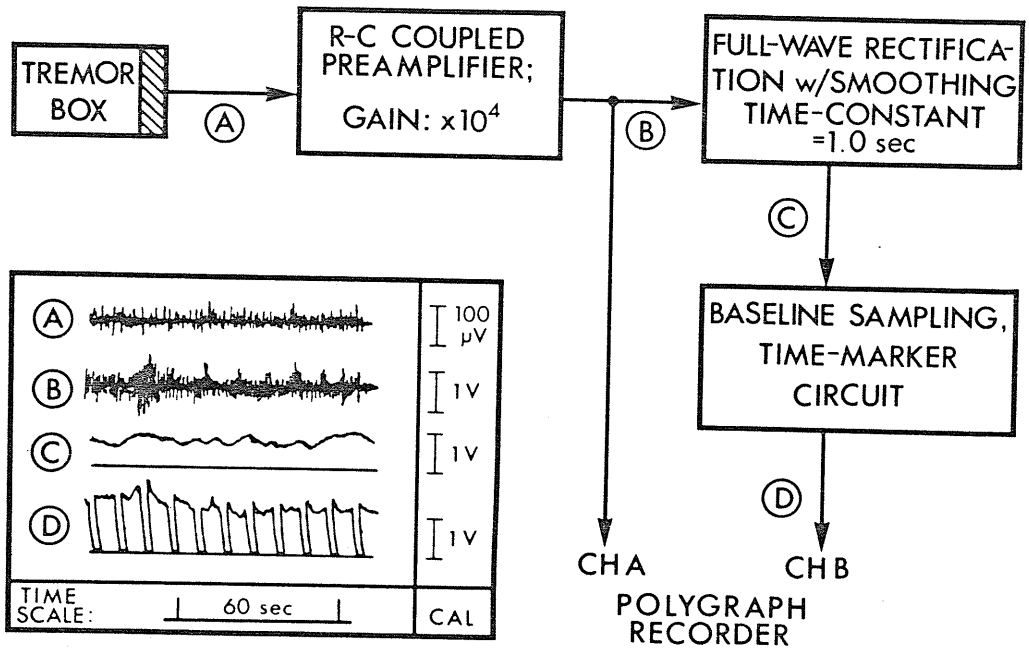

Fig. 4. Tremor Energy Analyzer; block diagram.

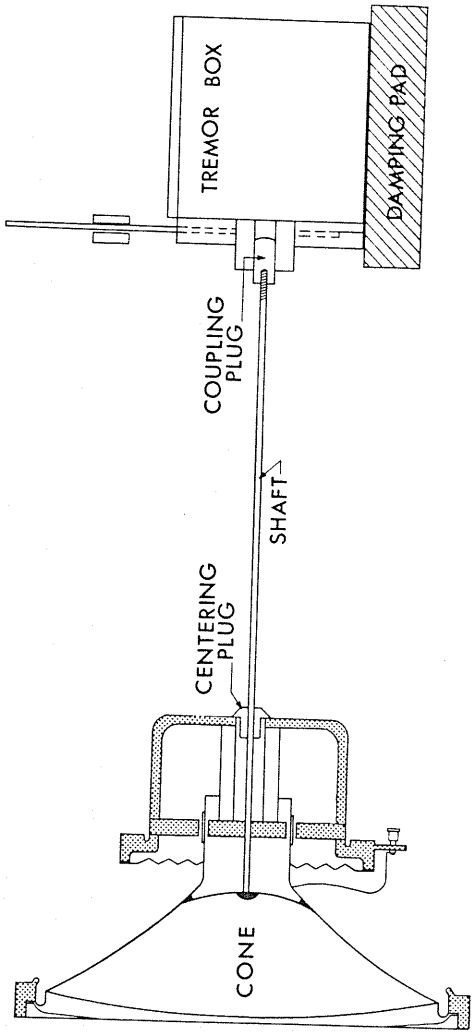

Fig. 5. Tremor Energy Calibrating System.

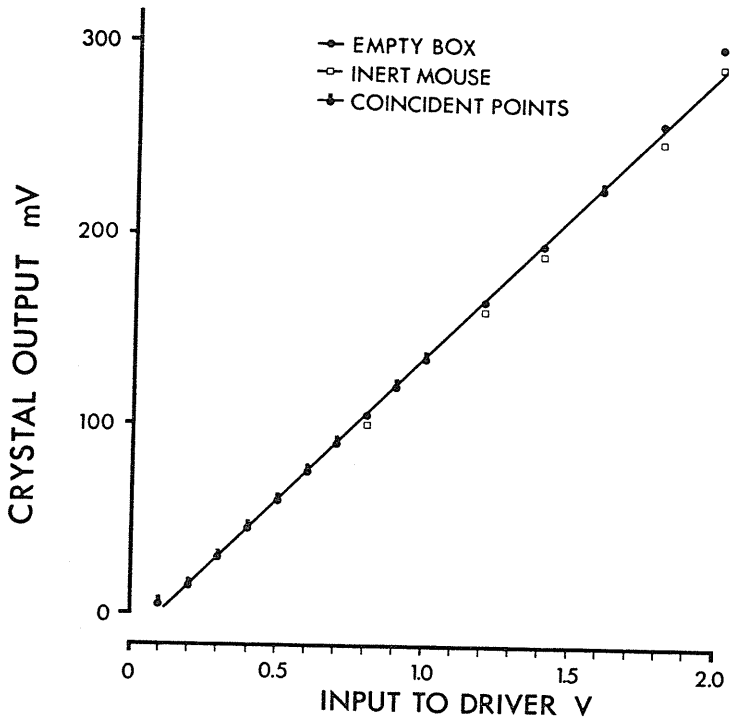

Fig. 6. Input-Output Transfer Function Curve, Tremor-box Transducer. Note that relationship remains linear and identical with constant mass, regardless of possible reactive virtual mass of live mouse.

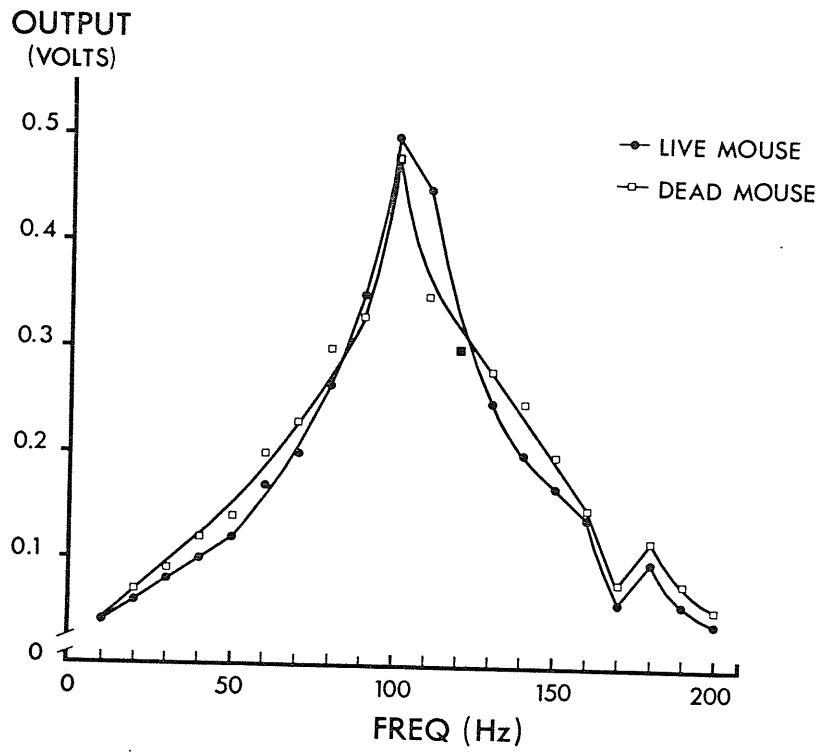

Fig. 7. Frequency Response Characteristics; Tremor-box Transducer. Note that resonant frequency remains essentially constant with constant mass, regardless of possible reactive virtual mass of live mouse.

existed a wide range of contributing frequencies and that to restrict the measurement range to 14-18 hz is in all likelihood incorrect and misleading. Also in Fig. 7 it can be seen that the frequency response over the lower range is a predictably uniform function and that movements from a live mouse do not affect this response to an appreciable extent.

In Fig. 3, a simulated tremor signal, consisting of a sinusoidal input of 700 μV RMS at 18 hz, has been presented to the amplifying-recording system to indicate the type of measurement made and the calibrating procedure used prior to the commencement of each experiment. The 18 hz frequency was chosen since, as mentioned earlier, preliminary studies indicated that this was a major component in the XTR-induced tremor signal. The upper trace shows the record of such a signal and the trace immediately below shows the output of the rectifying-averaging circuit. The height of the averaged trace was measured from the point at which the pen recording was interrupted ("A" to the zero datum line). The two lowest traces demonstrate the response of the unit to an abrupt change in the input signal. The average-rectified output incorporates the immediate past history of the tremor signal. The record asymptotes to the baseline within 3 seconds of abrupt removal of the input signal (bottom trace, Fig. 3).

(b) Injection procedures and tremor response measuremtns (tremorine and oxotremorine).

The following protocol was common to all tremor studies in which there was no immediate drug treatment, prior to administration of tremorine or XTR.

Female albino mice weighing between 20-25 mg were used. Animals

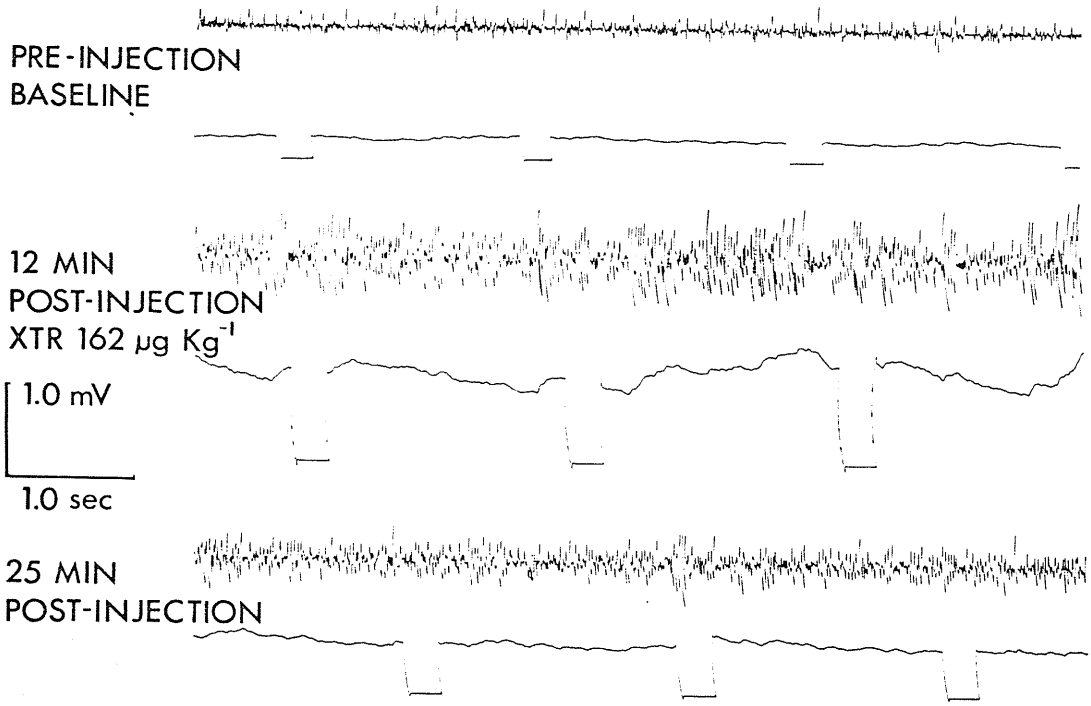

Fig. 8. Typical Procedures and Recordings; measurement of oxotremorine-induced tremor (see Fig. 3).

were placed in the tremor boxes for approximately 15 min before any recording was made. After this adaptation period, a baseline activity recording was monitored for 10 min (Fig. 8). Measurements were taken every 20 sec during the last 4 min. The average of these 12 numbers was taken as baseline activity, in arbitrary units. The animal was then removed from the box, injected i.p. with the appropriate drug and returned to the tremor box. These procedures interrupted the recording for no more than 30 sec, after which recording was immediately resumed. Measurements from this continuous record could be made at various time intervals, but were typically made at 20 sec intervals and averaged over every 4 min. Fig. 8 shows the response to an injection of XTR at 12 min post-injection and 25 min post-injection. In the latter case it can be seen that tremor has subsided considerably. The quantitated tremor response to a given dose of the drug was taken to be the difference between the mean value of the preinjection baseline measurements and the mean value of the post-injection measurements for a given 4 min interval.

These values were then analyzed by computer, using a program which eliminated outliers using the criteria for skewness and coefficient of variation of 1.5 and 20, respectively (Appendix). This program was used because it appeared that these outlying points of high amplitude in all likelihood represented either gross body tremor or the occasional poking and scraping movements referred to earlier in this section. Such outlying activity would therefore be unrelated to the fine body tremor associated with the response to tremorine and XTR, and could justifiably be discarded by the program. In confirmation of this, the full dose-response curves which were obtained with the drugs displayed a smooth and classical log-dose relationship with reproducible ED50 values (See RESULTS; Fig. 19).

(c) Measurement of harmine-induced tremor. Preliminary experiments with harmine induction of tremor in mice indicated that the drug induced a coarse involuntary oscillation of the trunk, hindquarters, head and neck, at times resembling gross shivering activity. The frequency components of this kind of tremor were predominantly in a much lower range than those of XTR-induced tremor. Hence, averaging the tremor signal and smoothing it with a time-constant appropriate to its low-frequency content (as was done for the higher-frequency tremor characteristic of XTR - see ... 'Methods for measuring XTR tremor") would have yielded an unacceptably long response time. For this reason, an alternative method for quantitating the magnitude of tremor activity was used in the experiments with harmine-induced tremor. Tremor activity in the form of the amplified tremor signal provided by the piezoelectric transducer was registered on the pen recorder and relative values for the magnitude of harmine were assessed by comparing pre-injection and peak post-injection recordings of the tremor record, obtained within 30 min of drug injection, to sample segments which had been assigned scores of 1 to 10; these sample segments had been selected from preliminary studies and were collated on a single page in an increasing magnitude of tremor activity in order to facilitate comparisons (Fig. 9). All tremor values were based on the difference between pre- and post-injection tremor. Values were assigned to the experimentally-derived recordings by two independent observers who assessed the records in blinded fashion. The mean of their assessments of tremor for each dose of drug was used in plotting the data.

The quantitative validity of this method was verified by plotting the dose-response relationship for harmine tremor. The narrow limits

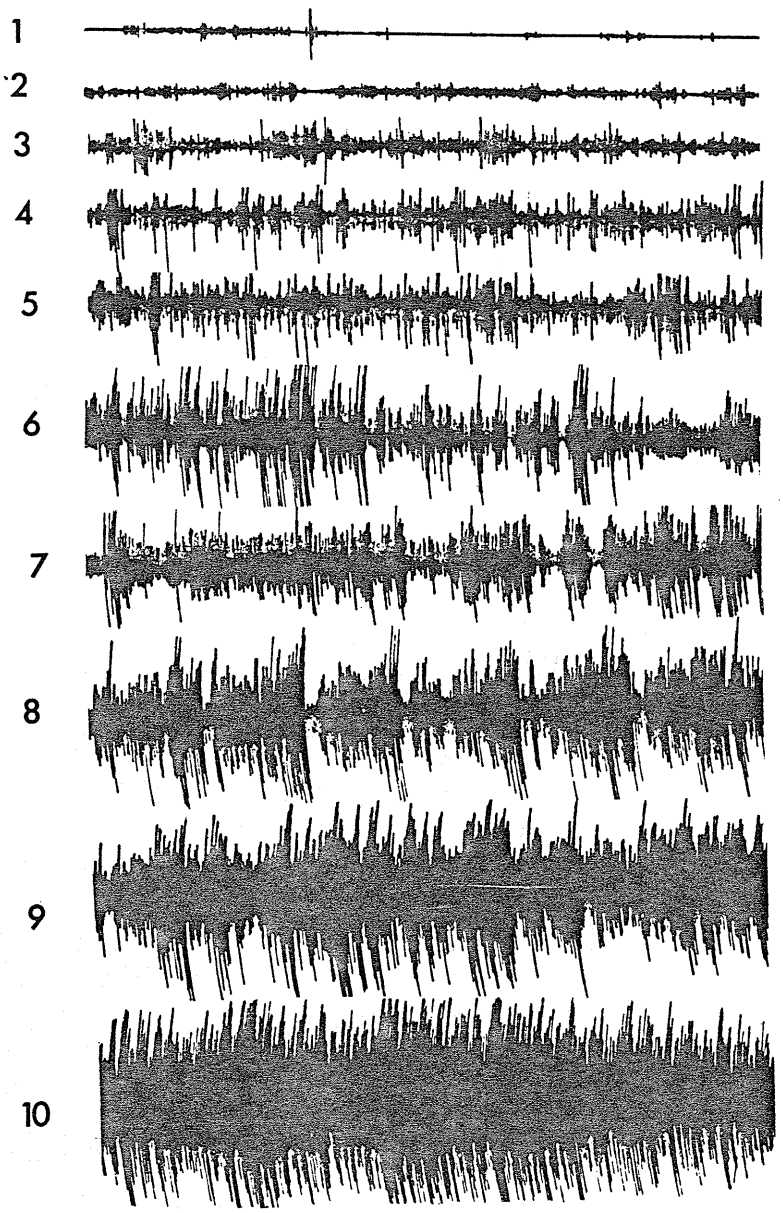

Fig. 9. Harmine tremor measurement scale.

of confidence thus obtained for the estimated ED50 of the drug implies considerable internal consistency of the procedure (Fig. 15).

(d) Dose-response studies.

(i) XTR dose-response curve. Solutions were so made that all injections were in the ratio of 0.1 ml to every 10 gm body weight. A large number of doses was used because of the initial indication of a very steep dose-response relationship. Doses of 58, 100, 132, 152, 175, 187, 200, 250, and 300 mg kg^{-1} body weight were used. Each group consisted of at least six animals except for the two highest doses. These latter were assigned fewer numbers because the tremor response began to fall off at these doses, presumably due to non-specific and toxic effects. Time-response relationships were observed over a 20 min post-injection period.

(ii) Harmine. Female albino mice weighing 20-25 gm were used for all tremor studies. Animals were housed in plastic cages in groups of 10-12 per cage and were maintained on food and water ad libitum under 12 hr light-dark lighting conditions. An acclimation period of several days was allowed before experiments commenced.

Harmine was dissolved in a 1% lactic acid solution in sufficient quantitiy to make all injectable volumes equal to 0.1 ml per 10 gm mouse weight. A total of 6 doses (5,10,20,30,50 and 80 mg kg^{-1}) in addition to lactic acid controls was used. Each dose was given to 12 animals for a total number of 84 (7 x 12) animals.

Animals were placed in the tremor box for a baseline period of approximately 10 min; they then received an injection i.p. and were observed for another 15 min minimum. During this entire period, a record of the mouses' activity was obtained from the pen recorder. The

The mean tremor amplitude ± standard error (S.E.) was calculated for each group and then used to determine the ED50.

(e) Drugs modifying tremor.

(i) XTR-drug interactions. To determine the cholinergic specificity of the tremor response, groups of animals were pretreated with 4.0 and 10.0 mg kg^{-1} atropine sulphate, 30 min prior to receiving their injection of XTR. Control groups received saline in lieu of atropine sulphate. Statistical analysis was done by Student's t-test.

(ii) Choline chloride effects on tremor and tremorigenic activity. Choline chloride, in several doses, was given to mice to determine its tremorigenic activity and its ability to decrease or enhance the tremor produced by XTR.

Choline was administered i.p. 30 min prior to injections of XTR or saline and the identical protocol for measuring tremor was subsequently followed. Doses of choline ranged from 20 to 150 mg kg^{-1} and those of XTR from 100 to 200 mg kg^{-1}. This range of doses was felt necessary because it was unknown at which dose the bimodal effects of choline chloride would be displayed. Consequently, although the combinations were numerous, they were necessitated by the lack of information which was available for the cumulative "cholinergic drive" which occurred in the CNS for XTR and choline chloride.

(iii) Harmine - drug interactions. Naive mice were pretreated with various drugs to determine which drugs blocked or potentiated harmine-induced tremor. The following drugs were used: propranolol 30 mg kg^{-1}; atropine 10 mg kg^{-1}; choline 50 and 55 mg kg^{-1}; morphine.

(f) Tremor sensitivity during chronic morphine administration.

(i) Cholinergic sensitivity during morphine dependency.

TABLE II

MORPHINE HABITUATION SCHEDULES

(intraperitoneal injections twice daily)

SCHEDULE mg kg^{-1}	DAY 1	2	3	4	5	6	7	8	9
'RAPID'	5	25	45	65	85	105	125	145	165
'SLOW'	5	10	15	20	25	30	35	40	45

Two groups of mice were made dependent on morphine by administration of this drug according to dose schedules which differed in the rates at which the doses were increased (Table II). A third group was handled similarly but was given saline in lieu of morphine to constitute the controls. Cholinergic reactivity was tested in the following way: at various times after receiving their day 9 injection, mice were placed in the tremor recording apparatus and the standard protocol was followed. Animals were injected with a small dose of XTR (100 mg kg^{-1}) because it was felt that an increased responsiveness would be more easily discerned from a low level of tremor than from a higher one. Also, preliminary studies had suggested that our instruments were sensitive enough to reliably detect small differences in either direction, even at this low dose.

Because of the nature of the results from the study just described it was decided to test more animals through an entire dose schedule using the "Slow" schedule of Table II. Different groups of animals were tested on da 2, da 5, da 7 and at 24 hours withdrawn after da 7; the data were then analyzed as previously described. At a later time, a second study was done in an attempt to check the reproducibility of our results.

(ii) Aminergic sensitivity. Animals were injected twice daily with MS according to the "Slow" schedule (Table II) or with saline (controls) and different groups were tested with harmine on da 3, 5, 7, and 9 and 24 hr withdrawn after da 9. In addition, one group was challenged with naloxone on da 9 to determine the severity of withdrawal while another group which had received saline for 9 da, was given an acute dose of morphine equal to that dose given to the morphine-dependent

mice on da 9. All animals were tested with harmine between 1 and 3 hr after their morning injection of morphine.

Tremor magnitude was determined as described previously for harmine and Student's t-test was used to determine statistical differences between groups.

(C) STEROID INVOLVEMENT IN MORPHINE ACTIONS

Male Sprague Dawley white rats, 140-160 mg, were prepared in three groups of nine each: (i) adrenalectomized (bilaterally); (ii) "steroidectomized" (bilateral adrenalectomy with gonadectomy); (iii) sham-operated. The experimental groups were maintained for 4-6 da on standard feed with 0.9% saline to drink ad libitum, the sham-operated group had feed and water ad libitum. Rats were placed on a hotplate at 44.5 $\pm$ 0.1^{o}C and observed for 15 min while the number of times they licked their paws was recorded. The 15 min experimental period was divided into 22 epochs of 1.0 min each; each epoch overlapped its predecessor by 20.0 seconds. The plotted data show the number of pawlicks counted in each epoch (Figs. 26, 28). As well, latency to the first instance of pawlicking was measured (Fig., 25, 27). Either saline or MS 5.0 mg kg^{-1} was injected i.p. 20 min prior to placing the rat on the hotplate.

Preliminary studies indicated that 44.5^{o}C was the threshold stimulus and all studies were then done at that temperature. The arrangement of the apparatus is shown in Fig. 10.

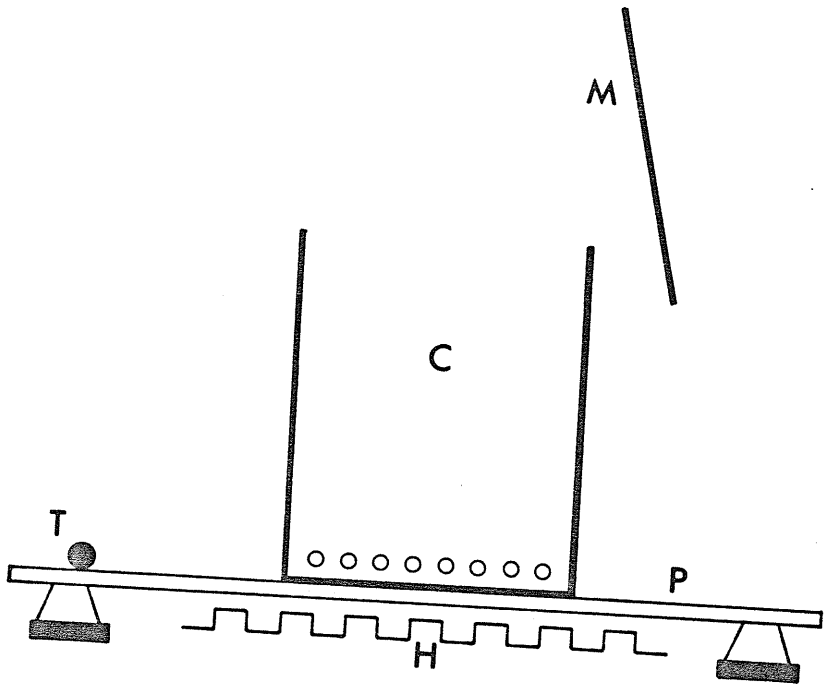

Fig. 10. Setup for measurement of paw licking, during sustained mildly noxious stimulation. T = thermostat probe (in practice close to cylinder, C); P = teflon-coated warmplate; H = regulated heater; M = observation mirror, to afford 360° visibility.

III RESULTS

(A) CHOLINE-MORPHINE STUDIES

(a) Antagonist-precipitated jumping as a measure of withdrawal in morphine-treated mice. The results of this study are shown in Table III. Nalorphine, a narcotic antagonist, 10 mg kg^{-1}, caused jumping in 7 of 8 morphine-treated mice (G. 1). This was significantly different from the group of mice receiving saline in lieu of morphine (Gp 3). Withholding the narcotic from morphine-treated mice (Gp 2) for 3 hr yielded only a small proportion of jumpers, not significantly different from zero. Nalorphine produced no jumping in the control animals (Gp 3) even over extended observation periods.

(b) The effects of choline on morphine withdrawal jumping in mice. The results of this study are shown in Table IV. The total number of surviving animals that received choline (independent of dosage) in the groups receiving MS-1 is 19. There is a significant difference in the proportion of jumpers in this group (8/19) compared to those animals not receiving choline (Gp 1; 7/8). Reduction of jumping in the morphine-dependent animals could not be attributed to choline-induced impairment of neuromuscular function since other motor activity (corner exploring, running) was not impaired. Moreover, we have never observed any motor impairment in other mice chronically given choline in doses larger than any used in the experiments described here.

Analysis of the individual groups that were given choline showed that there was one death in Gp 1 and two deaths in Gp 2 (See "Choline Treatment" in Section A of "METHODS"). The former occurred soon after the morphine injection but before the choline injection and therefore cannot be attributed to any choline toxicity. The two deaths in Gp 2, however, occurred soon after the choline injection on da 9 and were

TABLE III

NALORPHINE-INDUCED JUMPING IN MORPHINE-DEPENDENT MICE

GROUP	1	2	3
DRUGS INJECTED DURING HABITUATION	Morphine followed by saline	Morphine followed by saline	Saline followed by saline
CHALLENGING DRUG INJECTED 3 H AFTER LAST HABITUATING DOSE	Nalorphine	Saline	Nalorphine
PROPORTION JUMPERS*	7/8	6/16	0/6
SIGNIFICANCE OF DIFFERENCE FROM GROUP 1	-	$P \leq 0.025$	$P \leq 0.005$
SIGNIFICANCE OF DIFFERENCE FROM GROUP 3	$P \leq 0.005$	N.S.	-

* numbers jumping/total in group

TABLE IV

EFFECTS OF CHOLINE TREATMENT DURING MORPHINE DEPENDENCY, ON WITHDRAWAL JUMPING IN MICE

GROUP	1	2	3	4
DRUGS INJECTED DURING HABITUATION	Morphine followed by saline	Morphine followed by saline	Morphine followed by choline	Saline followed by saline
CHALLENGING DRUG INJECTED 3 H AFTER LAST HABITUATING DOSE	Nalorphine	Saline	Nalorphine	Nalorphine
PROPORTION JUMPERS Numbers jumping / Total in group	7/8	6/16	14/39	0/6
SIGNIFICANCE OF DIFFERENCE FROM GROUP 1	-	$P \leq 0.025$	$P \leq 0.025$	$P \leq 0.005$
SIGNIFICANCE OF DIFFERENCE FROM GROUP 4	$P \leq 0.005$	N.S.	N.S.	-

preceded by convulsive activity similar to that seen in those animals in the acute choline toxicity study. In contrast to this toxic effect, the high dose of choline achieved on this schedule appeared to be the optimal one with respect to the decreased jumping seen in those animals.

To obtain some indication of the effectiveness of choline in reducing withdrawal jumping but at the same time account for its safety in doing so, we chose to multiply the proportion surviving the study by the proportion of animals not jumping. This value is referred to hereinafter as the "Figure of Merit". The higher the number, the more favourable is the combination. The highest possible Figure of Merit, of course, is 1.0.

On average, it appeared that choline chloride in divided doses (C-3 and C-4; see Tables I and V) was the better method of administering this treatment, since this yielded Figures of Merit = 0.70 (Table V). Nevertheless, in those animals receiving MS-1 (Gps 1, 2 and 3, Table V) the single dose regimen C-2, ending at a single daily dose of 150 mg kg^{-1} choline chloride, gave a higher Figure of Merit (=0.63) than the C-3 regimen which ended in divided doses of 120 mg kg^{-1} twice daily (Table I) and yielded a Figure of Merit = 0.5 (Table V). It appears therefore, that moderately high doses of choline given just before withdrawal are more effective than lower doses, regardless of how they are given.

The control group (i.e. Gp 6, not receiving choline, Table V) had a Figure of Merit of only 0.13 which indicated that these animals, although not dying, suffered a severe withdrawal.

(c) The effects of choline on morphine withdrawal jumping in rats. Both morphine-dependent groups, with or without choline treatment, were significantly ($p \leq 0.01$) different from the controls, in proportion of

TABLE V

THE EFFECT OF CHOLINE CHLORIDE
ON SURVIVAL AND WITHDRAWAL JUMPING IN MICE

Group[a]	Morphine Regimen[a]	Choline Regimen[b]	Proportion Living	Proportion Not Jumping	Figure of Merit
1	MS-1	C-1	0.875	0.43	0.38
2	MS-1	C-2	0.75	0.83	0.63
3	MS-1	C-3	1.00	0.50	0.50
4	MS-2	C-4	1.00	0.70	0.70
5	MS-2	C-4	1.00	0.70	0.70
6	MS-1	none[c]	1.00	0.13	0.13

[a] see: METHODS; "Effects of choline on morphine withdrawal in mice". Habituation to morphine", pp. 41

[b] see: METHODS; "Choline treatment", p. 42

[c] received saline in lieu of choline; see note[b].

TABLE VI

EFFECTS OF CHOLINE TREATMENT DURING MORPHINE DEPENDENCY, ON WITHDRAWAL JUMPING IN RATS

GROUP*	PROPORTION JUMPERS	JUMPS PER ANIMAL
MS-N	15/23	6.9 ± 0.8
MC-N	7/11	2.7 ± 0.8
MS-S	0/8	0
SS-N	0/8	0
SS-S	0/8	0

* see: Methods; "Effects of choline on morphine withdrawal in rats", p. 42

leapers during precipitated withdrawal (Table VI). The two dependent groups were, however, significantly ($p \leq 0.05$) different from each other as regards proportion of leapers, with the choline-treated group showing about 60% fewer leapers than its saline-protected control (Table VI). Moreover, of those rats that did jump in these two groups, there was a significant decrease ($p \leq 0.01$) in the number of jumps in the choline-treated group, indicating that although these animals were experiencing withdrawal, the severity of this syndrome was significantly reduced by the choline. The morphine dependent group that received saline in lieu of antagonist (MS-S) displayed no jumping activity, indicating that these animals were receiving sufficiently high doses of morphine to keep up with their rates of development of tolerance. Additional support for their state of tolerance comes from the fact that no animals died from these high doses of morphine despite the ability of such doses to kill animals when given acutely (morphine lethality data; Fig. 11,12).

In addition to jumping, these withdrawing animals displayed a constellation of other signs, the most prominent of which were motor in nature. In particular, rearing, wet-dog shakes, "rim-walking", hole-probing, and increased exploratory behaviour were quite consistent from animal to animal during the precipitated withdrawal.

(d) LD50 for morphine in rats. The percentage of animals dying at each dose of morphine is shown in Fig. 11. The LD50 and the range of values for the LD50 $\pm$ SE are 493 and 455 to 529, respectively. Although a classical sigmoid curve has been drawn for these data, it can be seen, when a straight-line graph is constructed to join the experimental points (Fig. 12), that there is a slight drop in mortality at a dose of 550 mg kg^{-1}. This decrease is not significantly different but is interesting in light of the findings of Chesler et al. (1949), who saw a

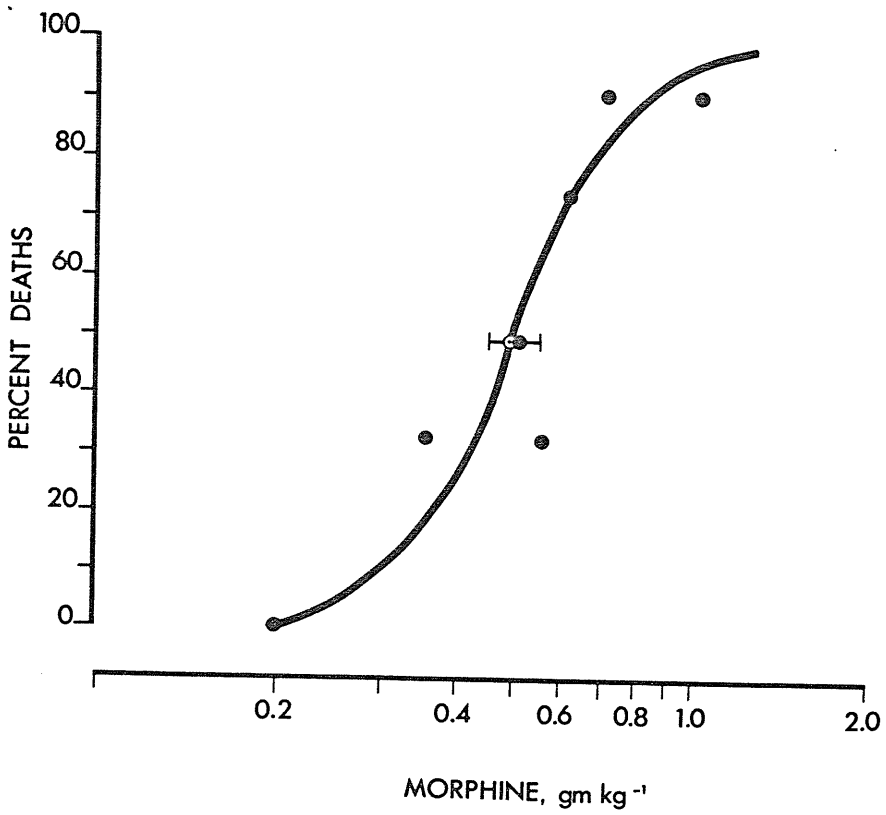

Fig. 11. Morphine lethality, best-fit curve. Horizontal bar at 50% lethality indicates LD50 95% confidence limits.

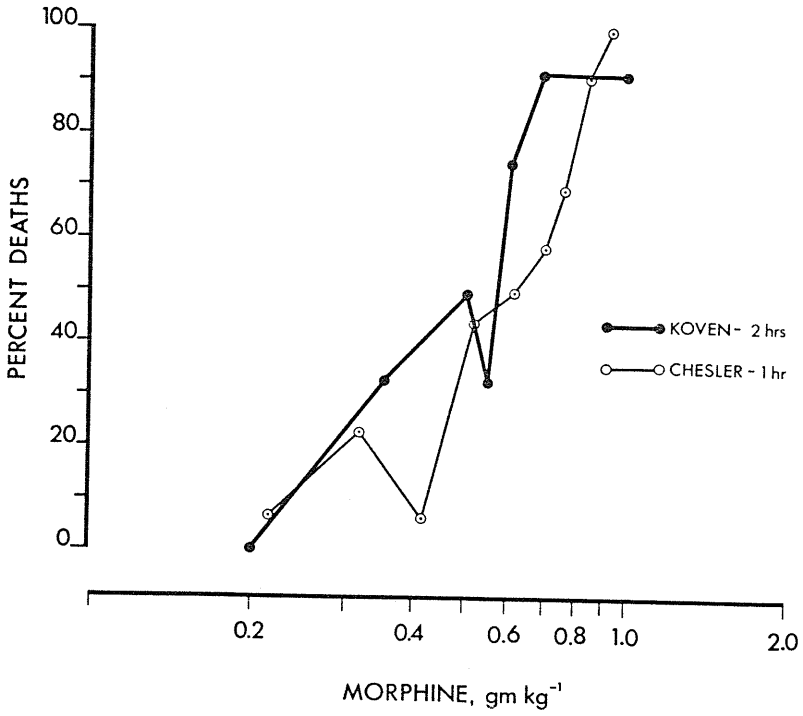

Fig. 12. Morphine lethality, unadjusted straight-line curve; comparison of Chesler's data with that from this study (see text).

similar drop in mortality at 400 mg kg^{-1} (Fig. 12).

Even at a dose of 1000 mg kg^{-1}, 100% mortality could not be achieved. The signs exhibited by these animals differed widely at the dose extremes. At the high doses, animals convulsed and jumped off the cage floor in a continual manner. At lower doses, animals pushed their noses through the spaces on the cage floor and clenched their teeth around the metal bars. They did so with such force that bleeding resulted. In the intermediate range, animals behaved more like the low-dose animals but usually survived.

(e) Determination of choline toxicity.

(i) Acute toxicity of choline chloride given intraperitoneally in mice. Choline chloride, in the batch (single shipment) used to plot the LD50 curve, caused no deaths in the mice tested at doses below 202 mg kg^{-1} (Fig. 13). This was even after an observation period extended to 24 hr. All mice that died, with any dose of choline given, did so within 15 min of injection. With doses of 254 mg kg^{-1} or greater, death occurred within 1-2 min of injection. The LD50 for choline was approximately 350 mg kg^{-1} (Fig. 13). Choline-induced death was always preceded by convulsive twitching of the extremities, with the animal lying fixed in one location. Urination and defecation occurred during the convulsions.

(ii) Cumulative toxicity. Although these animals were injected over a period of only 36 hr, some important conclusions can be reached from the data shown in Table VII. Ignoring for the moment the time of injection, as the number of choline injections (at 160 mg kg^{-1} each injection) increased, there was a minor but regular increase in lethality (Table VIIa). With one injection, 3/50 = 0.06 died; with two injections,

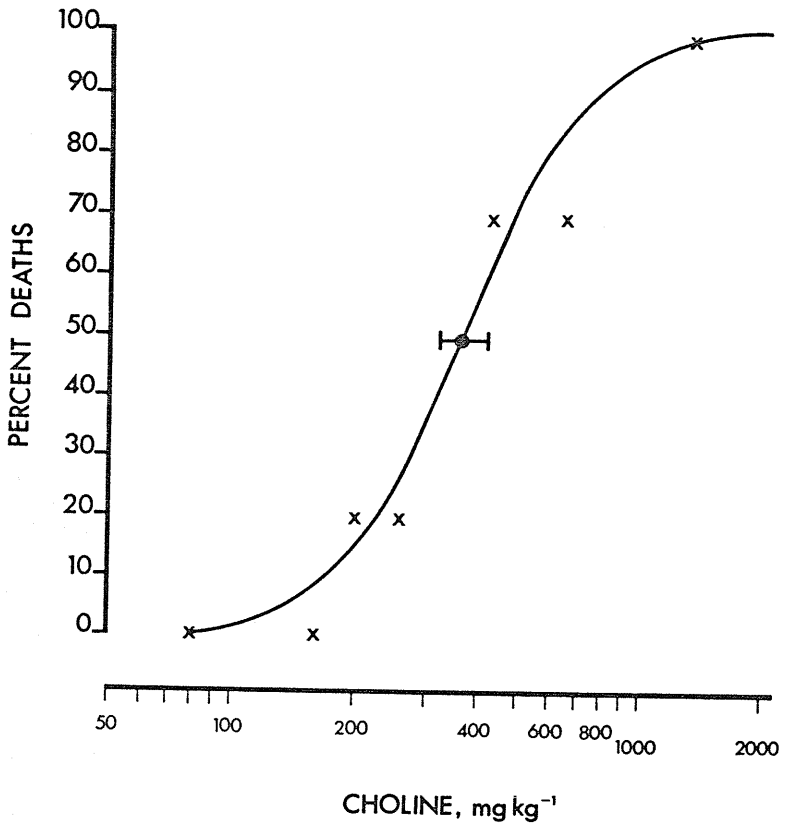

Fig. 13. Choline lethality, best-fit curve, statistical details as in Fig. 11.

TABLE VII

CUMULATIVE CHOLINE TOXICITY

(a)

	11 A.M. Day 1	11 P.M. Day 1	11 A.M. Day 2
Injection	Choline	Choline	Choline
Deaths	2/16	3/14	0/11
Injection	Choline	Choline	Saline
Deaths	0/10	3/10	0/7
Injection	Saline	Choline	Choline
Deaths	0/14	0/14	0/14
Injection	Saline	Choline	Saline
Deaths	0/10	1/10	0/9

(b)

Time of second injection of choline	No. of deaths
day	0/14
night	6/24

6/38 = 0.214 died. Those that survived two injections also survived a third. These data suggest that, when choline is to be readministered chronically, caution must be taken in the dose size administered and that the cumulative toxicity of the drug must be considered in its chronic use.

With respect to the toxicity as related to the time of administration, it appeared that the animals were more sensitive on their second injection at night, when they were on their dark schedule (Table VIIb). This may be related to the mechanisms governing diurnal fluctuation in brain ACh metabolism that has been reported by Hanin (1970).

(f) "Free" and "bound" ACh in morphine-dependent animals. Fig. 14 summarizes the results of the ACh extraction study. The data represent means and standard errors of ACh levels found in the three groups of animals studied (see METHODS pp 46-47). Total ACh levels were significantly higher in the dependent group as compared with the saline control and withdrawn groups (Fig. 14). Levels of "free" ACh in the dependent animals were significantly higher than in controls whereas this fraction of brain ACh had returned to control levels in the withdrawn group. Bound ACh levels were elevated in the dependent animals but were identical to control values in the withdrawn group. Ratios between the amounts of ACh in the different compartments were almost identical in the three groups.

(B) TREMOR STUDIES

(a) Harmine.

(i) Dose-response data. The dose-relationship obtained for the tremorigenic effects of harmine in drug-naive mice is shown in Fig. 15. It resembles in most respects, the results obtained by other investigators

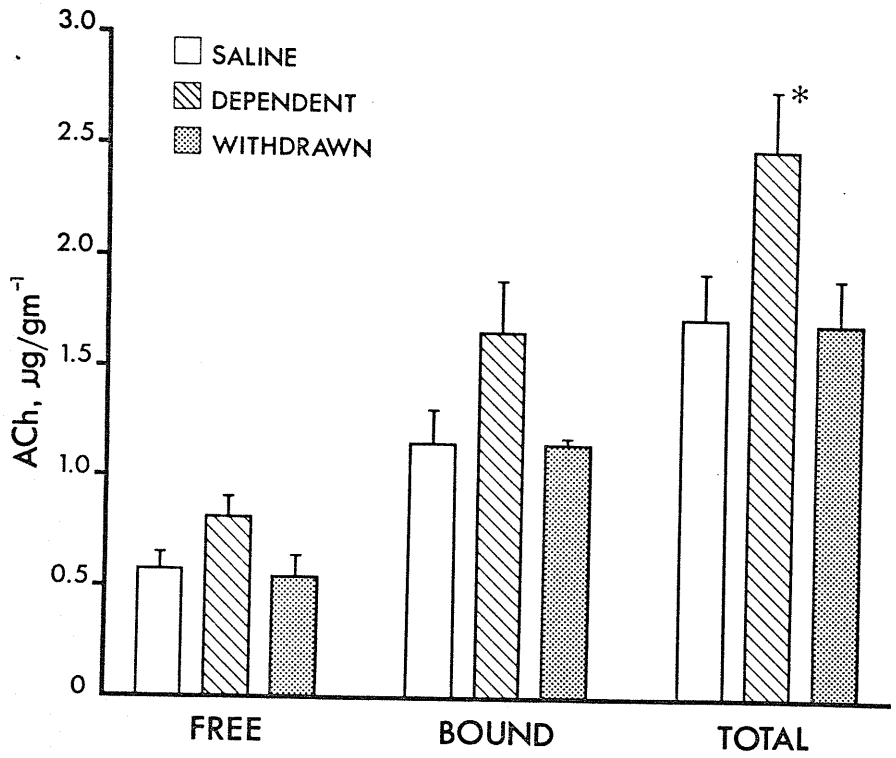

Fig. 14. ACh compartments in brain; variation with dependent and withdrawn states. * = different ($p \leq 0.05$) from "Total" values in "Saline" (control) and "Withdrawn" groups.

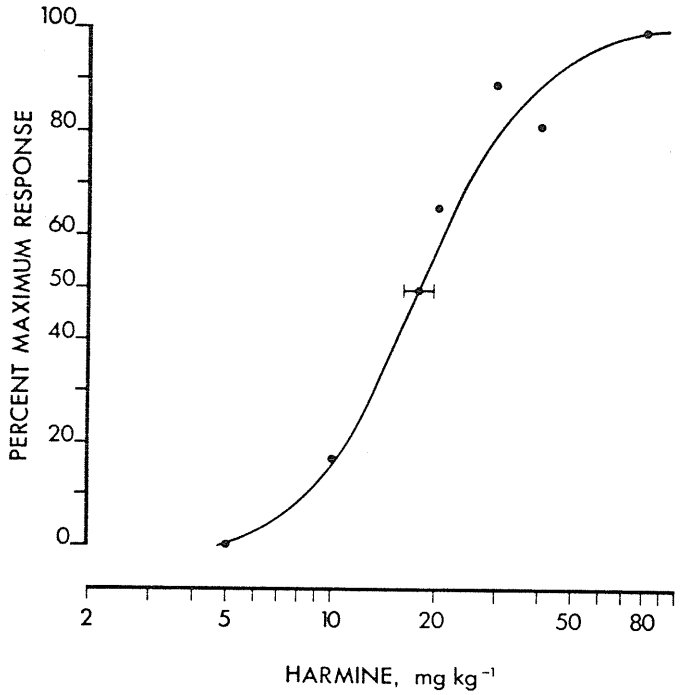

Fig. 15. Harmine tremor dose-response curve. Horizontal bar indicates ED50 95% confidence limits.

(Cox and Potkonjak, 1971; Kelly and Naylor, 1974). We found it necessary to use a dose higher than 40 mg kg^{-1} in order to obtain maximum tremor. A dose of 80 mg kg^{-1} was therefore used in addition to doses in the range that have been previously reported; this dose produced greater tremor but also increased the number of convulsions as compared to 40 mg kg^{-1}. The ED50 as computed from a program initially compiled for discrete data was 17.7 mg kg^{-1}, 95% confidence limits were 16.2 - 19.2 mg kg^{-1}. This range is acceptably narrow, and is close to the dose of 20 mg kg^{-1} specified by many other investigators.

In all subsequent studies with harmine a dose of 18.0 mg kg^{-1}, representing the nominal ED50 was used.

(ii) Harmine - drug interactions. Propranolol 30 mg kg^{-1} blocked harmine tremor (Fig. 16); atropine 10 mg kg^{-1} and choline 55 mg kg^{-1} failed to decrease the intensity of tremor although there appeared to be some alteration in the time course of tremor onset and duration. This contrasts with the results obtained with these latter two agents in XTR tremor (Figs. 21,22). Atropine blocked the tremor (Fig. 21) and choline chloride exerted a bimodal effect on it. At 50 mg kg^{-1} choline chloride significantly diminished the tremor response to 162 mg kg^{-1} XTR at both 12 and 16 min post-XTR injection (Fig. 22).

(iii) Sensitivity to harmine during morphine treatment and withdrawal. The tremor response obtained from mice which reveived saline for 9 da was consistently greater than the degree of tremor seen in naive mice given that dose. Hence, the ED50 for harmine must be different in naive and saline-treated mice.

It is difficult to account for this discrepancy, but extended acclimation to housing conditions and increased handling may be responsible

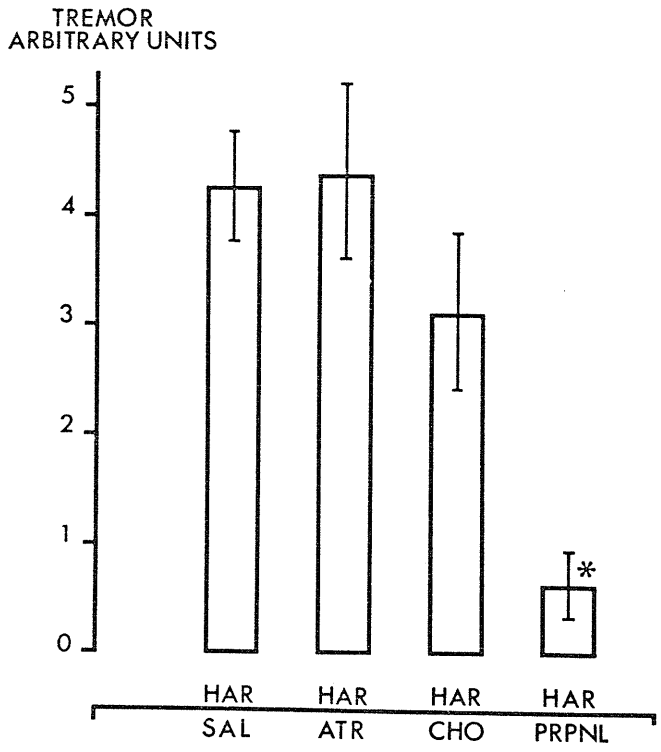

Fig. 16. Harmine-drug interactions. * = Significantly ($p \leq 0.05$) reduced as compared with harmine/saline group. HAR = harmine, SAL = saline, ATR = atropine, CHO = choline, PRPNL = propranolol.

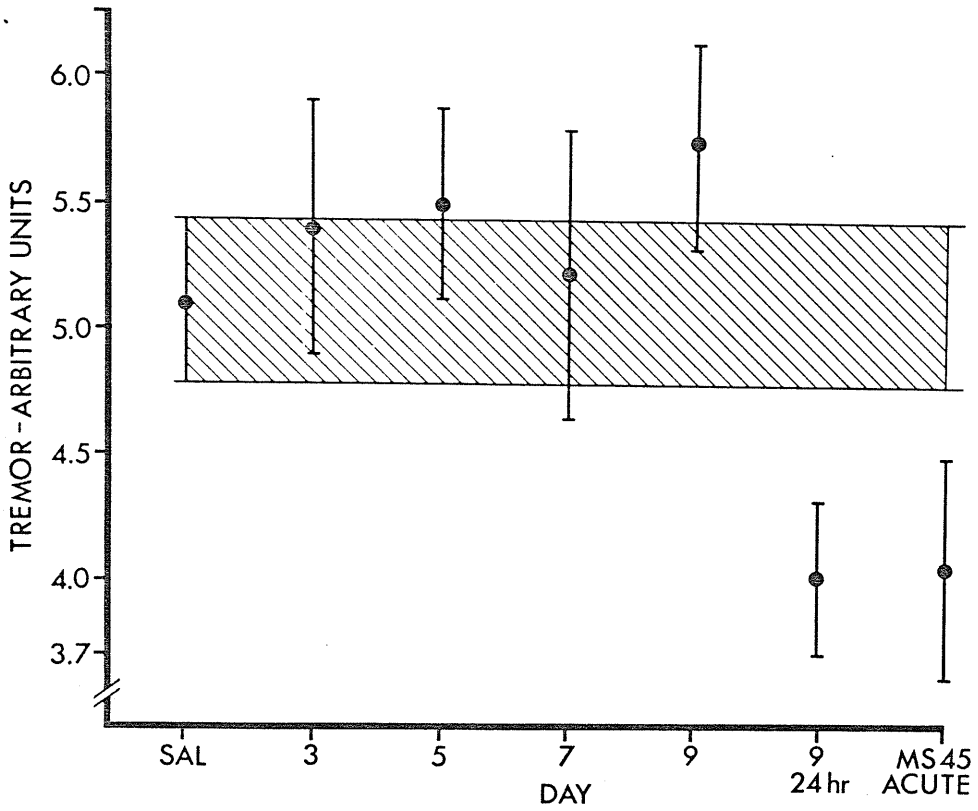

Fig. 17. Harmine tremor sensitivity during morphine dependency in mice. SAL = average for all animals given saline as control for each corresponding day in morphine habituation schedule. 9/24 hr = response to harmine after withholding morphine for 24 hr after da 9. MS 45/ACUTE = response to harmine in nontolerant mice, immediately after injection i.p. of morphine sulphate, 45 mg kg^{-1}.

for this increased sensitivity to harmine. Nevertheless this effect was consistant in mice regardless of the day of testing and all experimental groups were therefore compared to the control saline-treated group. There is no difference in sensitivity to harmine in mice maintained on morphine for 3,5,7, or 9 da (Fig. 17). There is a significant ($p \leq 0.05$) decrease in sensitivity to harmine in those mice which had not been given morphine for 24 hr after 9 da of injections (i.e. 24 hr withdrawn). There appeared also to be decreased sensitivity to harmine in those mice given an acute high dose of morphine after 9 da of saline injections (Fig. 17), although this decrease was not statistically significant.

Naloxone administration precipitated withdrawal jumping in 8 of 10 morphine-dependent mice but in no saline control animals. This high proportion of jumpers indicates that even the "slow" (MS-2) morphine schedule can produce a consistently high level of dependency.

(b) Oxotremorine and tremorine.

(i) Dose-response data. The XTR dose-response curve is shown in Fig. 18. Each point represents the mean value obtained by averaging the results from 6 to 20 animals. The slope of the curve was steep and the ED10, ED50, and ED90 values were 91, 132, and 188 $\mu g\ kg^{-1}$ respectively. At doses higher than 200 $\mu g\ kg^{-1}$ the response decreased, indicating that the tremor response may have been suppressed due to an increased toxicity of XTR at those doses. The two highest doses were omitted in calculating the parameters just specified. Even when they are included, however, the values obtained do not differ by more than a few percent.

The time-response relationship is shown in Fig. 19. At most doses, the response reached a peak at 12 or 16 min and then remained unchanged or had declined by 20 min.

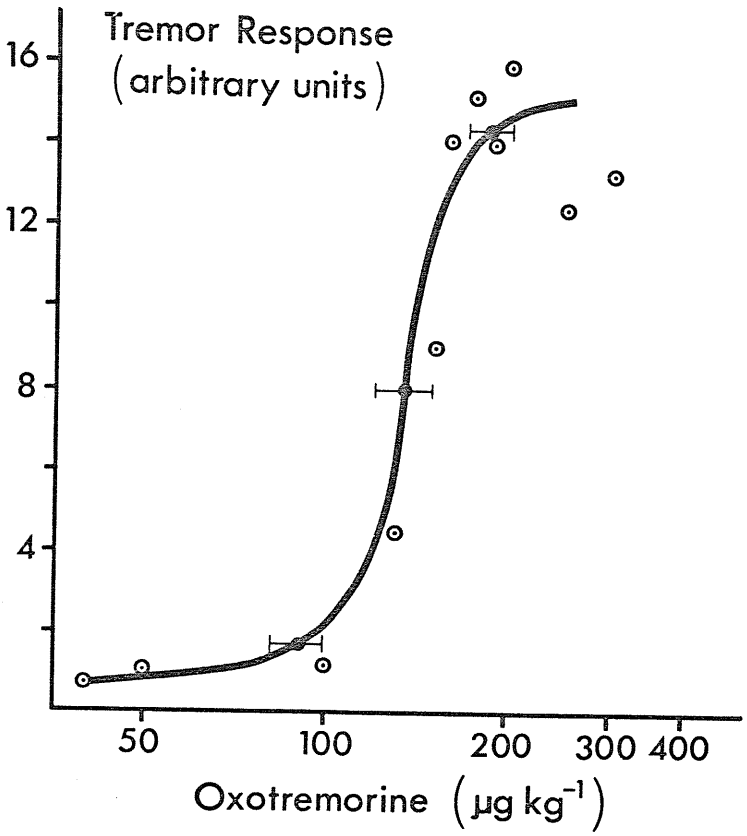

Fig. 18. XTR Tremor dose-response curve. Horizontal bars, from top downward, indicate 95% confidence limits for ED90, ED50 and ED10 doses.

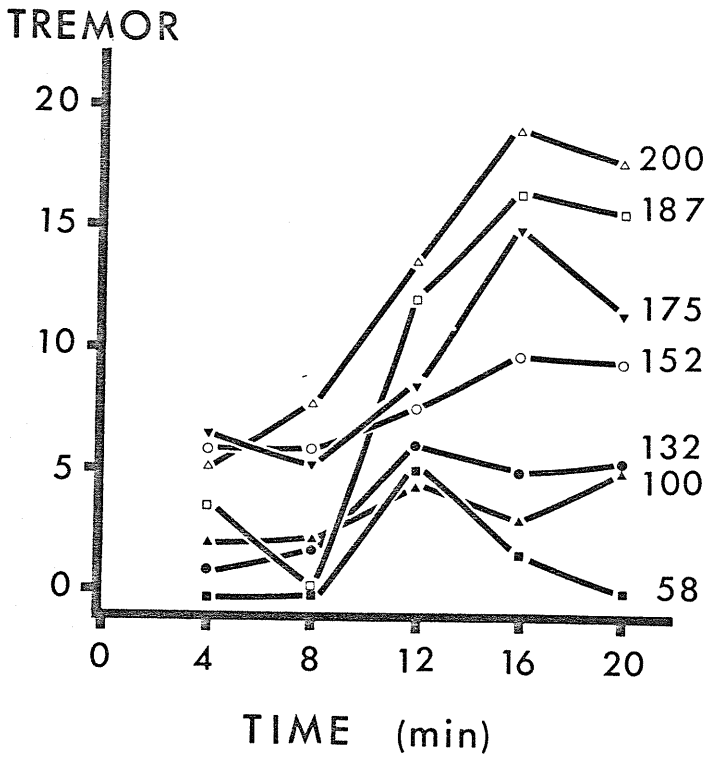

Fig. 19. XTR time-response curve. Numbers to right of curves indicate dose of oxotremorine sesquifumarate in $\mu g\ kg^{-1}$.

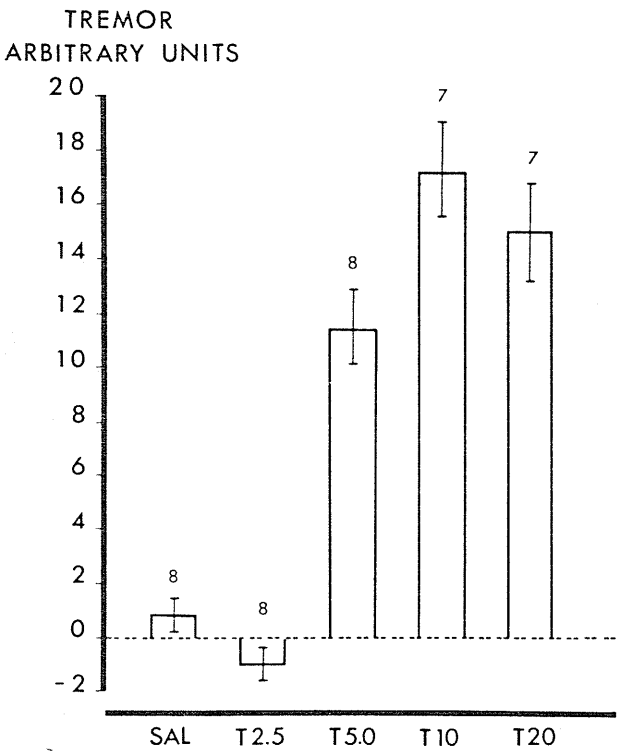

Fig. 20. Tremorine tremor responses. Numbers under each bar indicate dose of tremorine dihydrochloride (T) in mg kg^{-1}.

The data obtained with tremorine is shown in Fig. 20. The dose-response relationship agrees with those reported in the literature.

(ii) XTR - drug interactions. Pretreatment with atropine sulphate (Fig. 21) at a dose of 4.0 mg kg^{-1} decreased by approximately 60% the tremor response induced by XTR at a dose of 162 µg kg^{-1} but just failed to do so to a statistically significant extent. This may be attributed to the large variation seen in the saline pretreated control group. Significantly greater antagonism was found when atropine at a dose of 10 mg kg^{-1} was used. From these data it appeared that XTR was acting via central choline receptors although it cannot be entirely ruled out that there were not any peripheral actions as well.

Choline chloride, as a function of dose, either increased, decreased or had no effect on XTR-induced tremor. Fig. 22 shows the effects of choline chloride, at a dose of 50 mg kg^{-1}, on tremor. Choline by itself produced no tremor but, when used as a pre-treatment was effective in diminishing the tremor induced by XTR. At this dose it appeared to be acting as a cholinergic blocker in a manner similar to that shown for atropine sulphate.

(iii) Cholinergic sensitivity during morphine treatment and withdrawal. The results of an initial study, in which animals were not tested during the morphine habituation schedule but only after the end of the 9- day injection regimen, are shown in Fig. 23. Animals which received saline, in lieu of morphine for the 9 days, exhibited a small degree of tremor when injected with XTR at a dose of 100 µg kg^{-1} (as would be expected from the dose-response relationship shown in Fig. 18). The animals which had been injected according to the 'rapid' (Table II) schedule were slightly

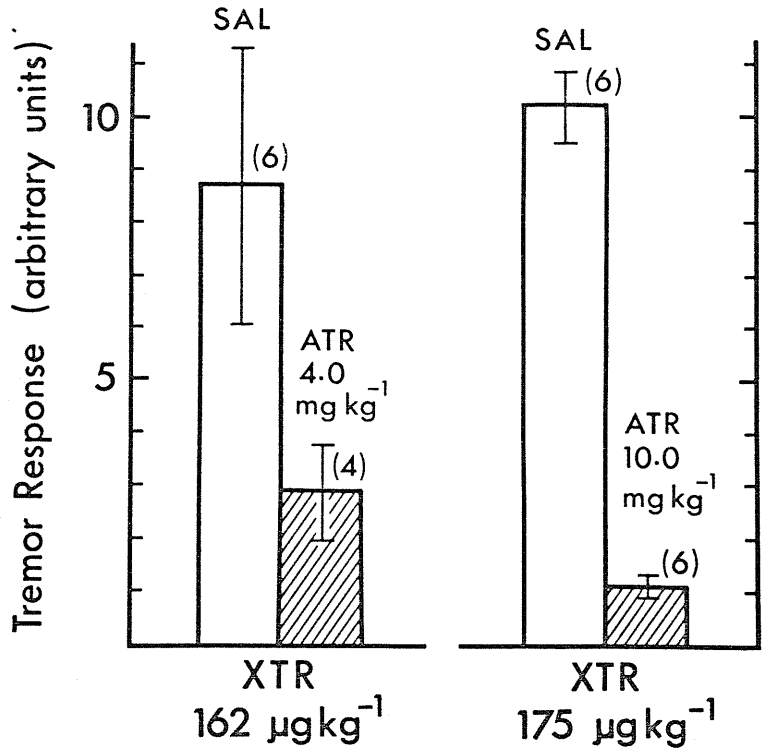

Fig. 21. XTR-atropine interactions.

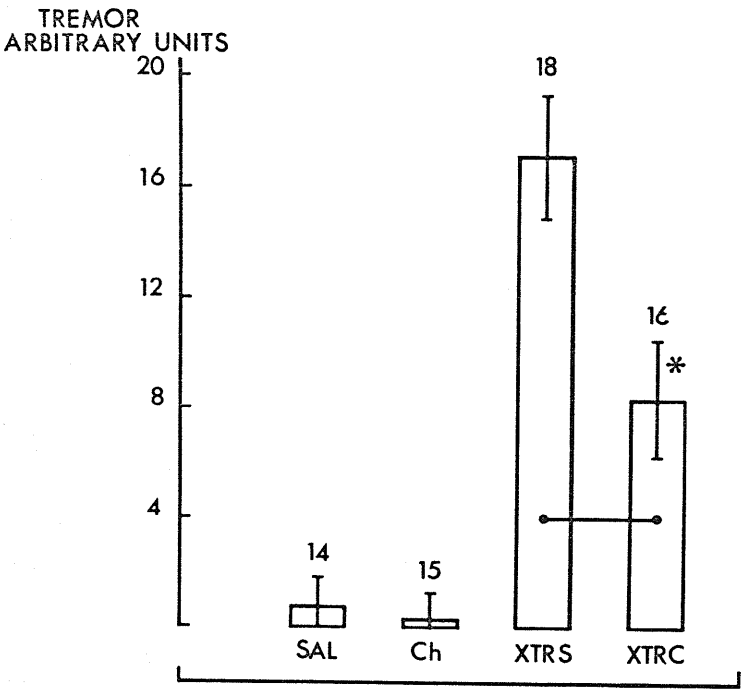

Fig. 22. Choline diminution of XTR tremor response.
* $p \leq 0.05$ difference between joined bars.

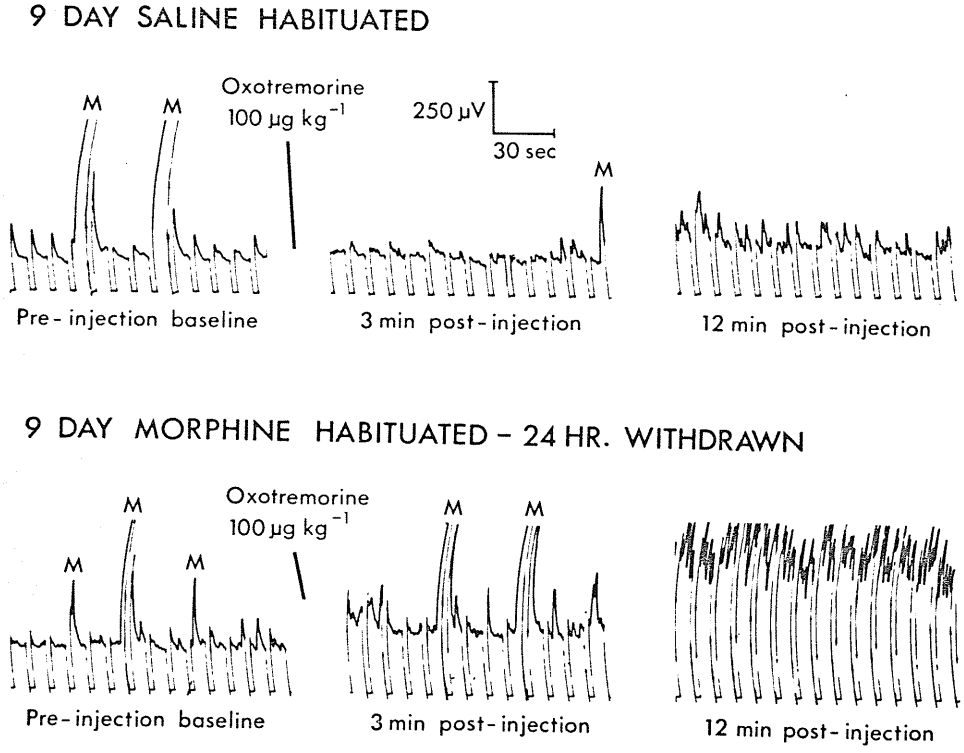

Fig. 23a. XTR responses in morphine-habituated mice. See caption, Fig. 23b, page 116

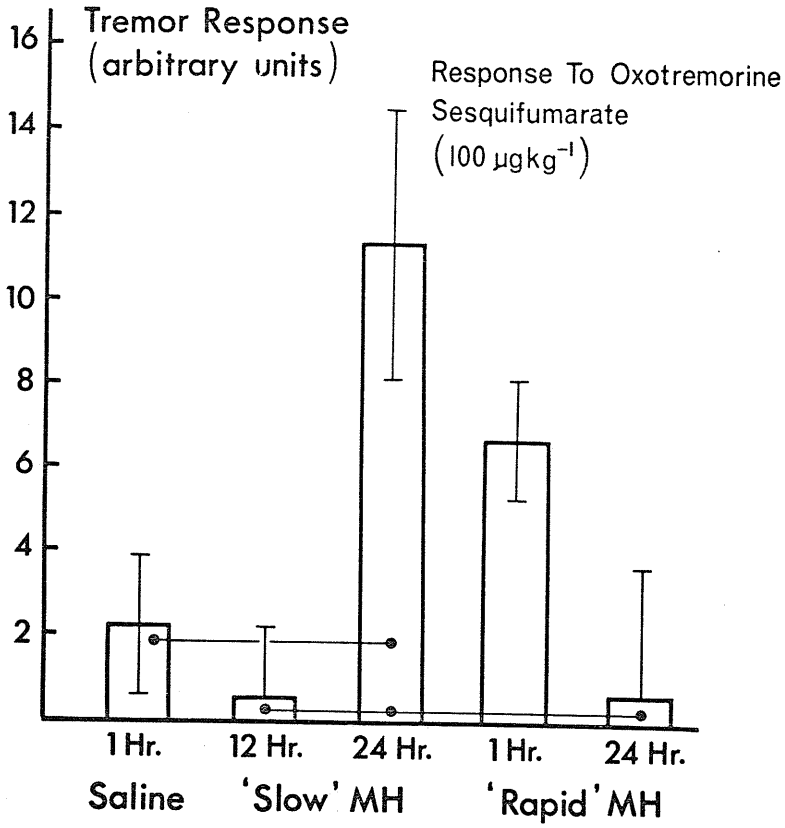

Fig. 23b. XTR responses in morphine-habituated mice. See "METHODS", Table II (p.86) for details of "Slow" and "Rapid" morphine habituation schedules. Responses are shown for times (1, 12 and 24 hr) after final injection. Treatments indicated in bottom line. Beaded lines join values that are significantly ($p \leq 0.05$) different from those at 24 hr after final injection of "Slow" morphine habituation (MH) schedule.

hypersensitive 1 hr after their last morphine injection but showed a return to normality 24 hr later. In contrast, those animals on the 'slow' schedule (Table II) did not exhibit this increased sensitivity until 24 hr after their last injection.

The results from the 'rapid' schedule could readily be explained according to the 'supersensitivity' model described earlier. However, no easy explanation could account for the other results. Consequently, in a further series of experiments, the tremor sensitivity was monitored throughout the entire morphine injection schedule.

Fig. 24 shows that the tremor sensitivity fluctuated phasically during the morphine injection schedule. There was an initial supersensitivity early in the schedule (da 3) which later disappeared. There appeared to be a rebound sensitivity during withdrawal both on da 7 and da 9, the latter to a significant degree. Hence there appeared to be an ongoing balancing phenomenon which took some time to establish but could be easily disrupted by altering the cholinergic drive during withdrawal.

(C) STEROID INVOLVEMENT IN MORPHINE ACTION

Morphine (M) significantly increased latency ($p \leq 0.02$) to first paw-lick in the sham-operated rats (sh; Fig. 25), as compared to their saline-treated (S) controls. The narcotic had a greater effect in the adrenalectomized (Ad) rats ($p \leq 0.005$) but had no effect on latency in "steroidectomized"(St) rats (Fig. 25). Responses to sustained (15 min) mild hotplate (44.5^{o}C) pain are shown in Fig. 26. Morphine significantly reduced the area under the curve (measured over epochs 7-17, where paw-licking was greatest and locomotor excitement least) as compared with the saline control group in adrenalectomized rats ($p \leq 0.02$). The corres-

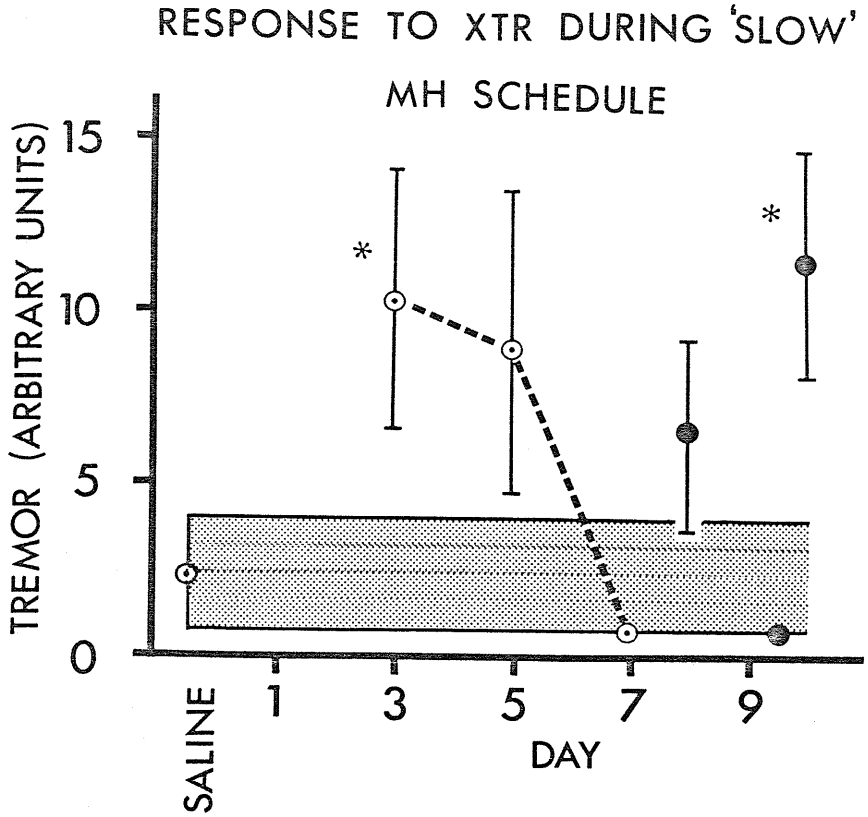

Fig. 24. Response to XTR during development of dependency to morphine habituation with "Slow" morphine schedule (see Table II). Extreme left-hand datum point obtained at 24 hr post-withdrawal. * $p \leq 0.05$ difference from saline-treated controls.

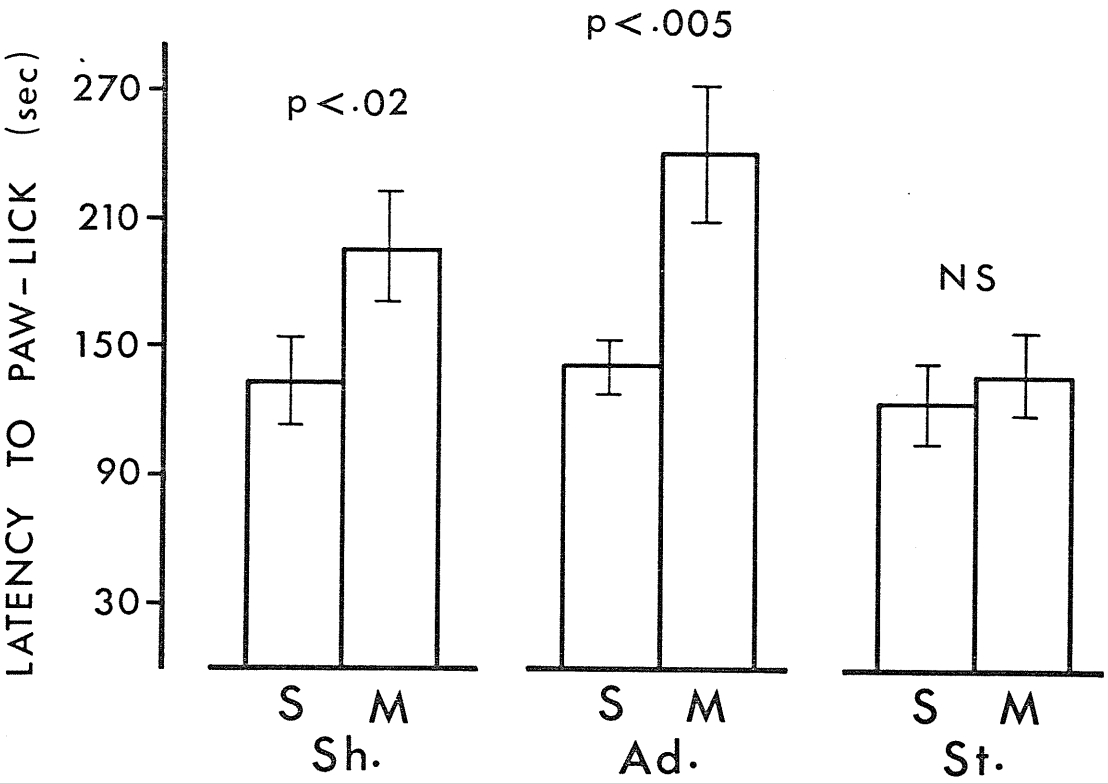

Fig. 25. Latency to paw lick in adrenalectomized (Ad.) and "steroidectomized"(St.) rats. "Sh" = sham-operated group. "S" = saline-injected, "M" = morphine-injected (5.0 mg kg^{-1}). NS = no significant difference between saline- and morphine-treated groups.

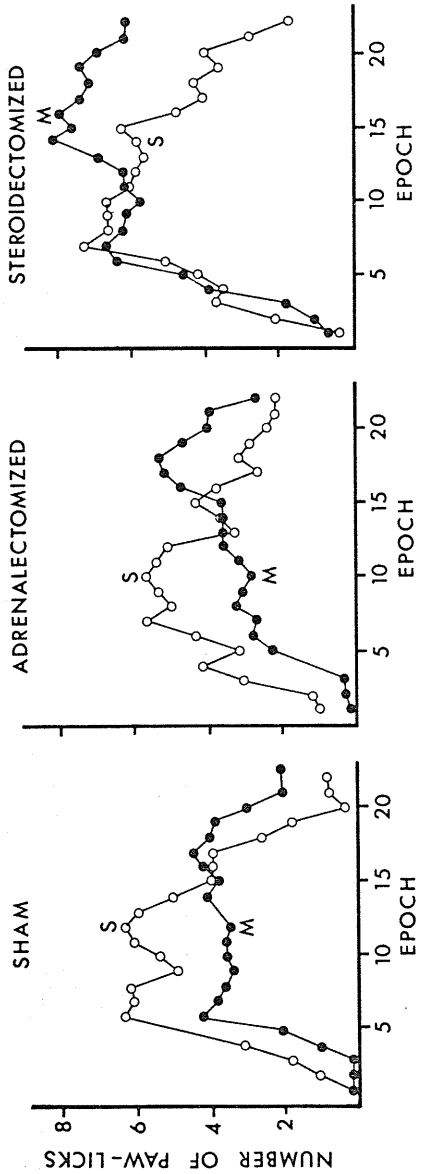

Fig. 26. Number of pawlicks per one-minute epochs in animals described in Fig. 25.

ponding reduction was not significant in the sham-operated group (Fig. 26). The narcotic slightly increased the corresponding area in the "steroid-ectomized" group.

In a second group of animals which were gonadectomized and unilaterally adrenalectomized, morphine (MS, 5 mg kg^{-1} i.p.) had no effect on latency to 1st hindpaw lick (Fig. 27) nor on the total number of paw-licks (Fig. 28).

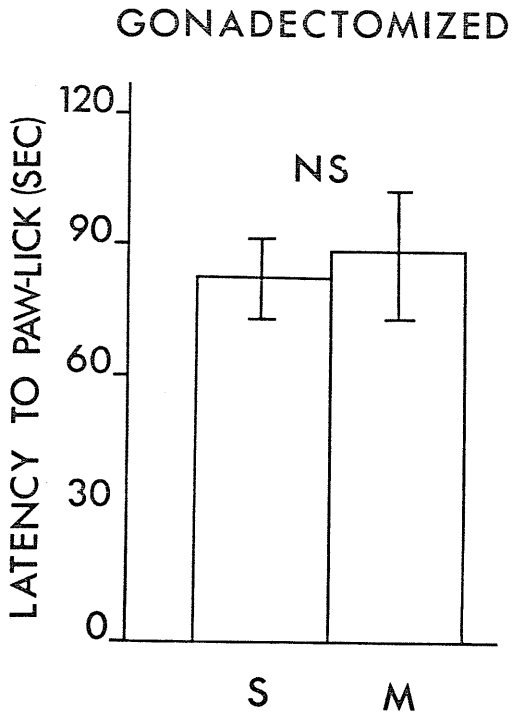

Fig. 27. Absence of morphine effects on pawlick latency in gonadectomized rats. "S"-saline-injected, "M" = morphine-injected (5 mg kg^{-1}).

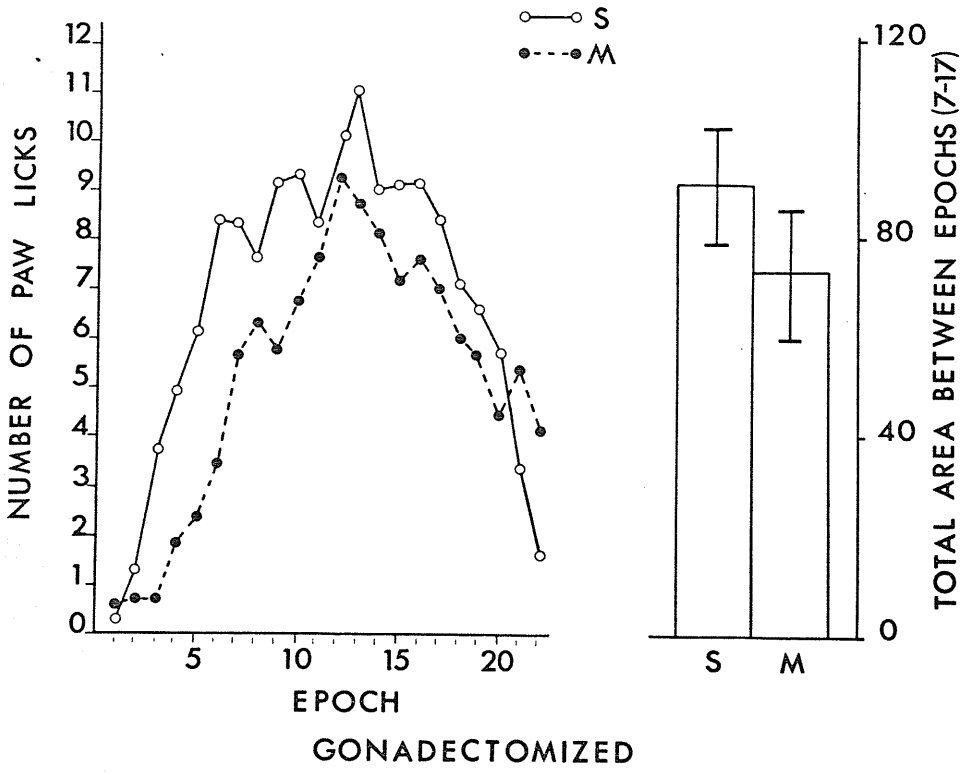

Fig. 28. Absence of morphine effects on number of pawlicks in gonadectomized rats described in Fig. 27. "S" = saline-injected, "M" = morphine-injected (5 mg kg^{-1}).

IV DISCUSSION

Cholinergic and Other Neurotransmitter Mechanisms in Morphine Dependency. This study has described the effects of morphine on a number of central cholinergic mechanisms and, as well, related these effects to the dependent state seen with prolonged administration of opiate narcotics. The possible involvement of an endogenous morphine-like substance in the mechanism of opiate narcotic dependency has also been considered and studied here. Such substances, unknown at the time when the bulk of this work was being done have now been identified (Hughes et al., 1975) and the postulation of their existence more than vindicated (see review by Frederickson, 1977).

The bulk of the work done in the present study was concerned with observations on central cholinergic sensitivity during the development of morphine dependency, and its possible modification by the partial cholinergic agonist choline. This writer's laboratory is not alone in postulating that morphine dependency may be due, in large part, to a derangement in central cholinergic mechanisms (Crossland, 1970; Jhamandas and Sutak, 1974; and Domino and Wilson, 1973a). However, although the just-cited authors support our view, there are others who disagree. Some have failed to discern any amelioration of the morphine withdrawal syndrome after the administration of anticholinergic drugs (Grumbach, 1969; Brase, 1974). Still others maintain that cholinergic upset, while contributing to the opiate narcotic withdrawal syndrome, is not the primary cause of dependency, but is only partly or indirectly related to the dependent state (Bhargava and Way, 1972; Collier et al., 1972). As well, the possible importance of cholinergic mechanisms has been denied by those investigators who maintain that the neurotransmitters

of likely importance in narcotic dependency are 5-HT (Way et al., 1969; Shen et al., 1970; Schulz et al., 1974) or the catecholamines, dopamine and noradrenaline (Schwartz and Eidelberg, 1970).

Much of the controversy regarding the relative importance of different central neurotransmitters in opiate narcotic dependency may reside in the complex interactions between the activity of neurotransmitter systems in the brain. In particular there is a growing body of evidence to show a reciprocal interaction between ACh and dopamine in the corpus striatum. The bulk of such evidence indicates that catecholamine release and turnover is stimulated by cholinergic neurons while the release and turnover of ACh is inhibited by dopaminergic drive (Anden, 1974; Stadler, et al., 1974). An interaction between ACh and noradrenaline in the brain has been less well-defined, but has nevertheless been discerned (Kazic, 1974) and judged as contributing to the hypertensive response to physostigmine. ACh-catecholamine interactions are further complicated by the possibility that ACh acts on central nicotinic receptors to cause a release of noradrenaline, while its central muscarinic effect is to inhibit the nicotinic-mediated release (Richardson et al, 1971). It is therefore not surprising that the administration of drugs which affect the function of one neurotransmitter system will affect also the activity of other neurotransmitters (Grewaal et al., 1974a; Goldstein, 1974) and that an upset in the function of any one central neurotransmitter will be reflected as changes in the activity of other central mediators. Chronically-administered morphine, therefore, might be acting to impair ACh release in the brain via an inhibitory dopaminergic mechanism (Lloyd et al., 1974; Grewaal et al., 1974b;

Merali et al., 1974; Grewaal et al., 1974c and Kuschnisky and Hornykiewicz, 1974) as well as by a direct effect on cholinergic nerve terminals. Conversely, morphine's ability upon acute administration to enhance the release of ACh in various regions of the brain (Mullin et al., 1973; Phillis et al., 1973; Mullin, 1974) might be the origin of that drug's apparent stimulation of cental dopamine release. There are but scant data to indicate which neurotransmitter system is acted on first in the sequence of neural events that follows morphine administration. Nevertheless, Wajda and her associates (1972) have reported that morphine administration in rats causes changes in brain levels of ACh, dopamine, norepinephrine, and serotonin; there were changes also in activities of the enzymes choline acetyltransferase and tyrosine hydroxylase. They found that the morphine-induced disturbances in the content of neurotransmitter and in the activity of the enzyme involved in its synthesis occurred more readily and extensively in the central cholinergic system while the striatal dopaminergic system responded much later to the administration of morphine. These latter data, at least, are in accord with the concepts put forward in this study, concerning the primacy of morphine's effect on the central cholinergic system in the development and expression of morphine dependency.

With regard to the role of cholinergic mechanisms in withdrawal jumping, it is of interest to consider that phenomenon as an exaggerated escape reaction in an animal submitted to an intense and adverse CNS upset. A considerable portion of normal activity in the strain of mice used here to study morphine dependency is spent in climbing, edge-running, leaping, and other rodent acrobatics. We have observed that,

during withdrawal, the mice will direct their leaps quite accurately if in doing so they can gain egress from a confined space. Hence, withdrawal jumping might be considered the animal's effort to utilize an inborn ability in order to escape a locale associated with a punishing stimulus. The data in Table IV indicate that treatment with choline during morphine habituation can diminish the ability of a narcotic antagonist to precipitate this specific sign of morphine withdrawal in mice. The absence of leaping in the habituated animals cannot be attributed to choline-induced impairment of neuromuscular function since other motor activity was not impaired. Moreover, we have not observed any motor impairment in other mice given doses of choline chloride equal to the largest that were used in the experiment described here.

It has been assumed, throughout this study, that the salutary effects of choline in preventing the development of morphine dependency, or in ameliorating the morphine withdrawal syndrome, are accomplished mainly via partial cholinergic agonism (Frederickson and Pinsky, 1975). As already discussed (see INTRODUCTION) choline's role as an ACh precursor might also contribute to the "anti-dependency" effect, by physiological antagonism (via nerve terminal loading) of the action which morphine has in the impairment of ACh release. The widespread distribution of ACh pathways in the brain suggests that several different systems, subserving quite diverse behavioural and motor functions, would be affected by a morphine-induced central cholinergic upset. This might explain why morphine withdrawal has such distinct and autonomic, dyskinetic and emotive components, and why choline has salutary effects on most of these.

It is known, for example, that stimulation of certain medial diencephalic structures can result in profoundly aversive behaviour; these structures seem to form part of a behavioural "punishment" system and appear to be cholinergically mediated (Olds and Olds, 1963; Stein, 1968). Kerr and Pozuelo (1971) have suggested that ventromedial hypothalamic structures, known to be part of a central mechanism for emotive behaviour (Brady, 1960) are importantly involved in the development of morphine tolerance and dependency. Wei and Way (1972) have observed that application of very small quantities of naloxone to medial regions of the thalamus can precipitate a stereotyped abstinence syndrome in morphine-dependent rats; many of the regions which responded to their localized naloxone stimulus are known to be richly innervated with cholinergic fibres (Brady, 1960). It is thus possible that withdrawal jumping is a behavioural expression of over-stimulation at cholinoceptive sites in the reward-punishment system, where only a small upset would be required to evoke widespread behavioural and autonomic derangement. Apparently choline, by virtue of its partial cholinergic agonism, is capable of preventing cholinergic overstimulation at sites in the morphine-dependent brain, that would have otherwise become supersensitive to central ACh drive. Since this is true of choline there is a strong argument to support the importance of cholinergic derangement as a major factor in the development of the morphine-dependent state, and not merely in its expression. The likelihood of a relationship between an upset in the limbic system, presumably the system in which the "reward-punishment" mechanism reside, to a dyskinetic syndrome such as compulsive antagonist-precipitated jumping, is emphasized by the reports of Kelly and Moore

(1976) and of Costall et al. (1976). These authors found that lesions in mesolimbic structures (nucleus accumbens, medial forebrain bundle) potentiated or induced dyskinetic circuling movements in response to parenteral amphetamine (a dopamine releaser) or to apomorphine (a dopaminergic agonist). They interpreted this data as indicating a mesolimbic influence on striatal activity. Upset in cholinergic pathways would thus influence the dopaminergic activities of both mesolimbic (and hence 'emotive') and neostriatal (and hence 'motor') activities. In fact, this relationship could specifically account for the peculiar combination of dyskinetic and autonomic signs typical of opiate narcotic withdrawal.

Labreque and Domino (1974) have described a complex sequence of inhibition and enhancement of ACh release from the neocortex of the cat undergoing withdrawal from morphine dependency. The series of events described there is highly suggestive of the under- and over-activity one would expect from a system of neurotransmitters whose net release is stabilized by various feedback loops involving mutual interaction between inhibition and excitation of activity in the different neurotransmitter systems involved in the development of dependency on morphine. Such cyclic changes in central cholinergic drive do in fact resemble the cyclic fluctuation in central cholinergic sensitivity observed during morphine dependency, as determined here by XTR agonism (Figs. 23, 24).

It has been demonstrated at the cellular level that central cholinergic supersensitivity may exist in morphine-dependent rats (Satoh, et al., 1975, 1976). The cited workers found that microelectrophoretically-

applied L-glutamate and ACh were more effective in stimulating single cortical neuron discharge activity in morphine-dependent rats than in drug-naive controls. The difference in threshold for ACh stimulation of the cortical neurons caused this putative neurotransmitter to become three times more effective in the dependent animals. Unfortunately, Satoh et al. (1976) did not attempt to antagonize the ACh stimulation with electrophoresed cholinergic blockers (e.g. atropine, mecamyline, or curare) and thereby characterize the nature of the supersensitivity. It is not likely however, that the apparent supersensitivity to ACh was due to a generalized hyperexcitability of neuronal membranes in the dependent animals, since the cholinosensitive cells were found (in contrast to glutamate-sensitive neurones) almost exclusively in deeper layers of the cortex (greater than 500μM below the pial surface; Satoh et al., 1975). In support of this, Yarbrough (1974) found that atropine was significantly ($p < 0.001$) less potent in blocking micro-electrophoretic ACh stimulation on cholinosensitive neurons in cerebral cortical neurons of morphine-dependent rats than in non-dependent controls. Hence, it appears that the selective central cholinergic supersensitivity observed by the effects of XTR on mice in this present study can be demonstrated directly in single neurons of rat brain.

Clinical Implications and Possibilities Resulting from this Work. We have shown that choline can act as a partial cholinergic agonist on central muscarinic receptors, as discerned by its interaction with the tremorigenic effects of XTR (Fig. 22). It is presumably this effect that accounts for choline's ability to prevent the development of morphine dependency in mice, as discerned by the jumping phenomenon (Table IV). The work done in this laboratory, both previously (Frederickson, 1971; Pinsky, et al., 1973; Frederickson and Pinsky, 1975) and as reported in

this present study, prompted a preliminary trial of choline chloride in human heroin addiction (done at methadone clinic, St. Boniface General Hospital). Although for reasons of small numbers of subjects the trial was not blinded, results suggested that choline was salutary in relieving the withdrawal sickness to some extent (Matas, Holms, and Pinsky, unpub.). Dyskinetic and gastrointestinal signs seemed to be most susceptible to choline relief. Should this work ever be repeated or extended, the following problems would have to be solved with regard to choline therapy: choline has a very short half-life in man, even after intravenous injection (Appleton et al., 1953), and its parenteral effects are short lived (Growdon et al., 1977). Hence, either a slow-release form of the substance should be administered (a pharmaceutical approach) or alternatively some other substances with partial cholinergic agonist properties and with longer-lasting parenteral effects could be administered. In seeking such a substance, e.g. by drug-screening procedures, it would be possible to use the technique and results arising from this study. Candidate compounds would be characterized in mice by testing the interaction of each drug, after intraperitoneal or oral administration, with the tremorigenic effect of XTR as described here. Evidence of partial cholinergic agonism would be confirmed by an interaction which resembles that predicted by Stephenson (1956) for interaction between full and partial agonists.

The ultimate objective in what has just been proposed would be to find a long-lasting central active partial cholinergic agonist which might be able to relieve narcotic dpendency with a single injection. Although this goal may seem ambitious it is nevertheless based on a

consistent rationale that appears to have been borne out in the studies with choline chloride that have already been reported from this laboratory and now extended by this present study. It is of more than passing interest to note that oral choline chloride is now being tested extensively in the treatment of both tardive dyskinesia (Davis et al., 1976) and Huntington's Disease (Growdon et al., 1977). It was informally suggested from this laboratory (personal communication between C. Pinsky and R.T. Ross) before the just-cited reports had been submitted, that choline should be tested for relief in Hungtington's Disease. The suggestion was based on observations of the excellent relief which choline had given against the dyskinetic components of opiate narcotic withdrawal in the experimental animals observed in this present author's study (pp 92-96) and in the patients observed in the preliminary clinical trial with oral choline chloride.

Mechanisms of Morphine Dependency at Loci Beyond Cholinergic Release. In searching for the molecular locus of morphine action, over the course of this work, this laboratory independently postulated the existence of a central endogenous morphinelike ligand for the stereospecific opiate receptr. Our initial hypothesis that this substance might be steroidlike has not been substantiated by chemical identification. Nevertheless, opiate ligands of a non-peptide nature continue to be described (Pert et al., 1976; Schulz et al., 1977; Blume et al., 1977), these are lipid-soluble and may yet prove to be steroids. Our experiments with gonadectomy, adrenalectomy and morphine antinociception have shown it likely, mild sustained pain may mobilize an endogenous steroid of gonadal origin that in some way acts to reduce nociception.

Whether this is by means of direct attachment to opiate receptors remains to be seen, but recent work with intracerebroventricularly-administered steroids from this same laboratory indicates that such a possibility cannot be ruled out (LaBella, et al., 1978a; LaBella et al., 1978b, 1979). Moreover, the results arising from that part of this thesis raise the possibility that the adrenal cortex, in response to mild pain stress, may release a substance which antagonizes the analgesic of endogenous antinociceptive steroids and of morphine as well. The possibility of a link between peptide endorphins and release of gonadal and/or adrenal steriods in response to pain has in fact been alluded to in very recent symposia (Goldstein, 1978).

Important to the total contribution of the work done in this thesis is the central hypothesis on which the steroid-related experiments were based, i.e. that an endogenous ligand of stereospecific opiate receptors might be physiologically-operative in adaptation ('accomodation') to sustained mild pain stress. It was this hypothesis (Goldstein, 1974) which prompted us to test the effects of naloxone on warmplate stress (Figs. 29,30) It is interesting to point out that an apparent analgesic effect of naloxone is seen with the lower dose (1.0 mg kg^{-1}) (Fig. 29) while practically no effect is seen at a dose tenfold higher (Fig. 30). This might be due to a neuronal regulation of central endorphin release. At the lower dose of antagonist, opiate receptors are blocked sufficiently to initiate central release via the release regulating system but not sufficiently to prevent the antinociceptive effects of the released endorphin. At higher doses of naloxone, however, there will be sufficient receptor blockade to prevent the antinociceptive effects of the feedback-stimulated release of endorphin.

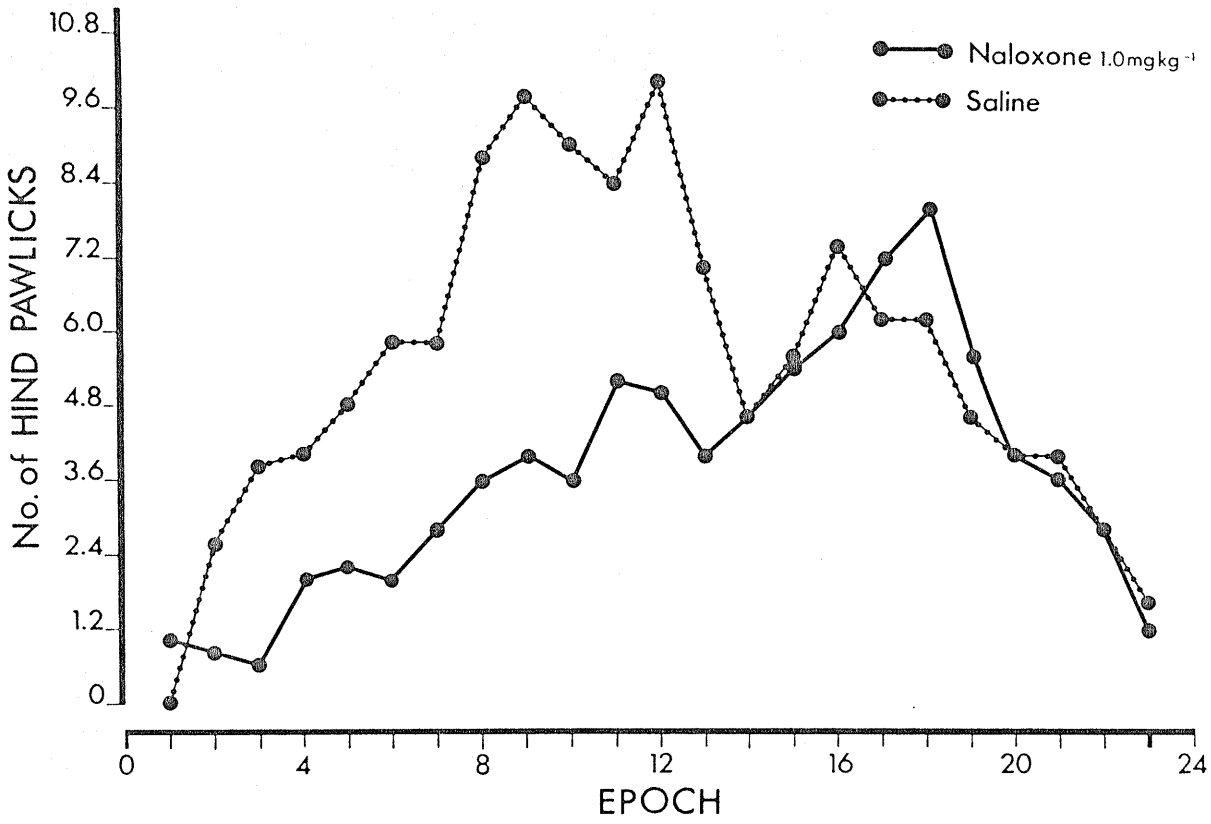

Fig. 29. "Anomalous" analgesic effect of naloxone hydrochloride, 1.0 mg kg^{-1}. This may represent one of the earliest demonstrations of "feedback" mobilization of endogenous opioid substance(s). Warmplate at 44.5°C.

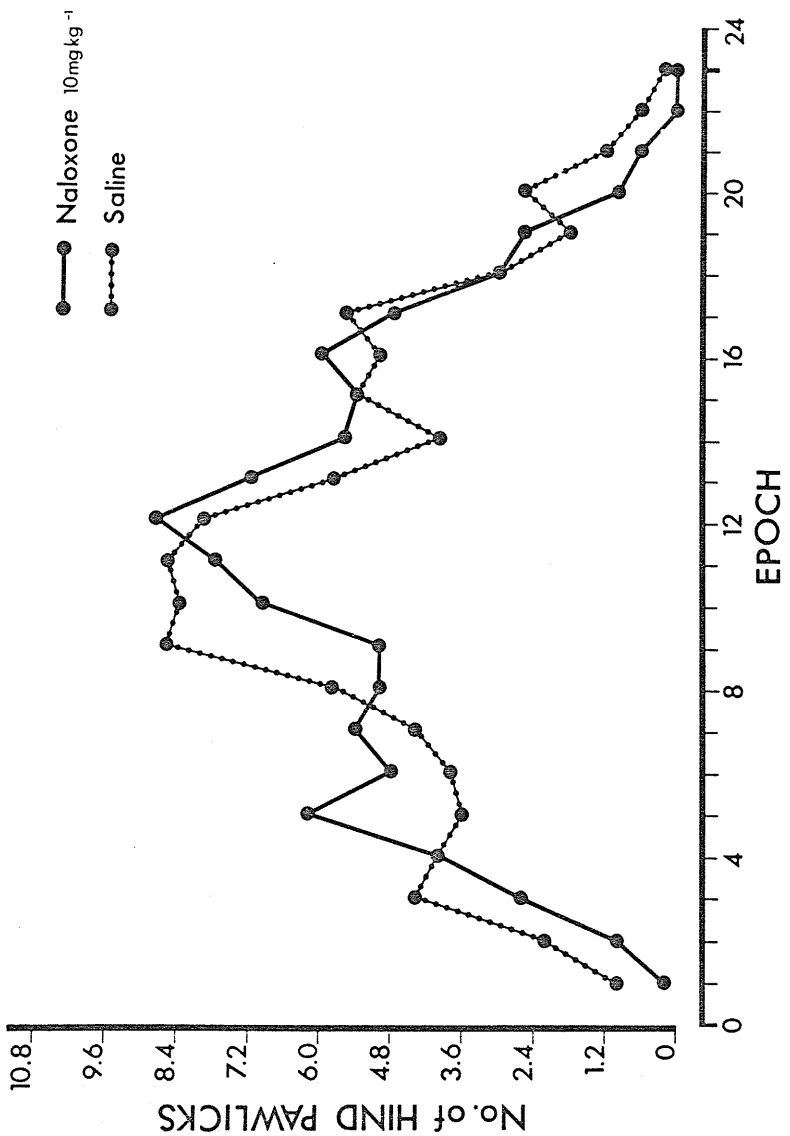

Fig. 30. Absence of either analgesic or hyperalgesic effect of naloxone hydrochloride at 10.0 mg kg^{-1} during sustained mildly noxious stimulus (warmplate at 44.5°C).

Such a mechanism predicts a rapidly-asymptoting dose-response curve, which has in fact been observed in other recent work in this laboratory (Pinsky et al., 1978). If the explanation just given proves to be correct for the anomalous antinociceptive effects of naloxone - an essentially pure narcotic antagonist - then the observation made in that section of this work represents one of the first demonstrations of a physiological role for endogenous ligand(s) of stereospecific opiate receptors.

V SUMMARY

The results of the work done in this study have shown that:

1. In the mouse, chronic morphine readministration induces a central cholinergic derangement, as assessed by the tremorigenic effects of parenteral XTR. Central cholinergic responsivity fluctuates cyclically from supersensitivity to subsensitivity during the dependent state, and exhibits considerable supersensitivity during withdrawal (Fig. 24). In contrast, no supersensitivity is exhibited to the tremorigenic effects of harmine hydrocholoride in mice on an identical morphine-readministration schedule. Thus, the upset in cholinergic sensitivity appears to be specific to morphine dependency, and not merely a manifestation of a generalized supersensitivity to all central neurotransmitters during a drug-dependent state. It is held, in the present study, that the crucial role of cholinergic derangement in morphine dependency accounts for the ability of choline chloride to impede the development of morphine dependency and to ameliorate diverse expressions (motor, emotive and autonomic) of opiate narcotic withdrawal sickness.

2. Choline chloride attenuates the repetitive upward jumping (characteristic of precipitated morphine withdrawal) in mice and rats. This amelioration of the opiate narcotic withdrawal syndrome in these two species was accomplished by administration of choline during the period of their habituation to morphine; this indicates that choline impedes the development of morphine dependency and does not merely suppress the expression of some specific sign of dependency. Moreover, since the effect of choline is similar in two different species, the results support the original premise that a species-independent cholin-

ergic mechanism is importantly involved in the development of morphine dependency.

3. Differing doses of choline chloride - in keeping with its expected ability to behave like a partial agonist on central muscarinic receptors - could increase, decrease, or leave unaffected, XTR-induced tremor in mice. At 50 mg kg^{-1} (i.p.) choline chloride antagonized XTR tremor, thus displaying an atropinelike effect at the dose just specified.

4. The acute and chronic toxicities of intraperitoneal choline will differ, because of cumulative toxicity with chronic administration. The LD50 for intraperitoneal injection (acute toxicity) is approximately 350 mg kg^{-1} (Fig. 11); the mice were more sensitive to choline toxicity in the dark (nighttime) portion of their 12-hr dark, 12-hr light schedule.

5. Chronic morphine readministration increases both "free" (readily releasable) and "bound" (storage pool or 'reserve') levels of ACh in rat brain; the percentage increase of each fraction is approximately equal (Fig. 12). Total brain ACh and its fractional distribution returns to control levels within 24 hours of withholding the drug to induce the withdrawal syndrome. The latter observation is consistent with the initial premise in this work, i.e. that the prime target of opiate narcotic derangement of central cholinergic transmission is at the level of presynaptic mobilization and release.

6. In seeking a molecular basis for the anti-ACh release action of morphine it was postulated in this study that the stereospecific opiate receptor would be involved. Extrapolating from a suggestion by Goldstein (1974), an endogenous ligand for the opiate receptor was sought in this work. The hypothesis made here was that such a substance

might be a gonadal or adrenal steroid. Although this has not been confirmed, such a possibility cannot be ruled out; allusion to the possibility remains in current literature (Goldstein, 1978). The work done in this study with regard to the effects of gonadectomy and adrenalectomy on responses to a sustained mildly noxious stimulus remains compatible with the possibility that there may be certain species of steroids acting as endogenous ligands of opiate receptors. Alternatively (or, as well,) nociceptive and/or antinociceptive steroids may form a chemical axis of activity on opiate receptors during the release of peptide endorphins in response to noxious stimulation.

7. In the course of examining the hypothesis concerning endogenous morphinelike substances, the effects of the opiate antagonist naloxone was tested on the response to a sustained mildly noxious stimulus in rats. The drug was observed to exert an anomalous antinociceptive effect at a low, but not at a high, dose (Fig. 29,30). The result is explicable on the basis of a feedback-provoked release of endorphin. This result represents one of the earliest experiments done anywhere to show an interaction between an exogenous opiate antagonist and a deliberate experimental provocation of endogenous opioid release.

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VII APPENDIX

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C-PFOCAL  CVSK@@FC

01.10 C C TTY:,E
01.15 E
01.20 C---CALCULATES CV & SKEW
01.30 C---DELETES MAX. VALUE UNTIL DESIRED CRITERIA ARE OBTAINED
01.40 C---CV CRITERION=20; SKEW CRITERION=1.500
01.50 A !"SAMPLE SIZE (N) :  "N
01.60 T %6.04;F I=1,N;A ":"X(I);I (FITR(I/7)-I/7)2.1;T !
01.65 C---TYPES DATA ONTO TAPE
01.70 O R C ;T %3,N;F I=1,N;T X(I)
01.80 C C TTY:,E
01.85 D 2;D 5.6;D 5.7;I (CV-20)1.87,1.87,10.1
01.87 I (SK-1.500)5.72,5.72,10.1

02.10 C---CALCULATES FOUR MOMENTS:MEAN,SD,SKEW,& KURTOSIS
02.20 C---MEAN=M1,SD=SD,SKEW=SK,KURTOSIS=KU
02.30 S X1=0;S X2=0; S X3=0;S X4=0
02.40 F I=1,N;S X1=X1+X(I);S X2=X2+X(I)↑2;S X3=X3+X(I)↑3;S X4=X4+X(I)↑4
02.50 S M1=X1/N;S M2=X2/N;S M3=X3/N;S M4=X4/N
02.60 S U2=M2-M1↑2;S U3=M3-3*M1*M2+2*M1↑3
02.70 S U4=M4-4*M1*M3+6*M1↑2*M2-3*M1↑4
02.80 S SD=FSQT(U2);S CV=(SD/M1)*100;S SK=FSQT(U3↑2/U2↑3);S KU=U4/U2↑2

03.10 C---DETERMINES IF CV MEETS CRITERION
03.15 C---IF IT DOES, CHECKS IF SKEW IS OK
03.20 C---IF IT DOES NOT,GOES TO 10.1 &DELETES MAX.
03.30 C---IT THEN REDOES 2
03.40 I (CV-20)4.1,4.1,10.1

04.10 C---DETERMINES IF SKEW MEETS CRITERION
04.20 C---IF IT DOES,GOES TO 5.1,IF NOT,DELETES MAX BY 10.1 ETC.
04.30 I (SK-1.500)5.1,5.1,10.1

05.10 C---TYPES DATA THAT HAVE MET CRITERIA & SAVES THEM ON DEC TAPE
05.20 C---TYPES MEAN SD CV SKEW &KURTOSIS
05.25 T !!!"DATA USED FOR FINAL ANALYSIS
05.30 T !%6.04;F I=1,N;T " "X(I);I (FITR(I/7)-I/7)5.1;T !
05.40 D 1.7;D 1.8
05.60 T !!"    MEAN          CV         SD       SKEW    KURTOSIS
05.70 T !%6.04,M1,%7.04,CV,SD,SK,KU
05.72 T !!"ARE THERE MORE DATA FOR THIS FILE--Y/N?";S X=FIN( )
05.74 I (X-206)5.72,5.75;I (X-217)5.72,1.5,5.72
05.76 C C
05.80 T !!!;QUIT

10.10 C---ELIMINATES MAX VALUE
10.15 S ML=1;F I=2,N;I (X(ML)-X(I))10.2,10.3,10.3
10.18 G 10.3
10.20 S ML=I
10.30 C
10.40 S X(ML)=X(N);S N=N-1
10.50 I (N-1)10.6,10.6,10.7
10.60 T !!!"THE DATA ARE TERRIBLE;I GOT RID OF THEM ALL";QUIT
10.70 G 2.1
*
```

Fig. 1 appendix. FOCAL program printout for statistical analysis of XTR tremor responses.