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A BIOCHEMICAL AND TOXICOLOGICAL COMPARISON OF T.2636 AND MORPHINE. (U)

[redacted]

BY

L. LEADBEATER.

CHEMICAL DEFENCE EXPERIMENTAL ESTABLISHMENT

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DATE: 16th December, 1963.

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by

L. LEADBEATER

SUMMARY

1. After the repeated administration of T2636 to rats the animals became tolerant to its toxic effects and to those of morphine. Similarly, morphine tolerant rats were found to be tolerant to T2636.

2. The in vitro N-demethylation of both compounds by the liver microsomal enzymes was depressed in preparations from both T2636 and morphine tolerant rats.

3. After withdrawal of T2636 from tolerant rats the pharmacological response of the animals to the drug and the liver microsomal N-demethylating activity returned to normal within 25 days.

4. N-allyl normorphine, an antagonist of the pharmacological actions of both T2636 and morphine, inhibited the N-demethylation of both compounds.

5. It is concluded that T2636 produces its biochemical and pharmacological effects by a similar mechanism to morphine.

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INTRODUCTION

The chemical structure and pharmacological activity of T2636, 6:14-endoetheno-7-(1-hydroxy-1-cyclohexylethyl) tetrahydro-oripavine, are similar to those of morphine (1). The present investigation was undertaken to ascertain whether the biochemical activities of the two compounds are similar and whether T2636 produced its pharmacological effects in the same way as morphine.

Morphine is N-demethylated *in vitro* by liver microsomal enzymes, in the presence of $NADPH_2$* and oxygen, to give normorphine and formaldehyde (2,3). Although this reaction has only recently been shown to occur *in vivo* (4,5) it has important pharmacological implications because it may be related to the mechanism of action of morphine (6) and to the development of tolerance to the drug (7). The following similarities have been observed between the CNS receptors of the morphine-narcotics (i.e. those sites in the CNS with which they interact to produce their pharmacological effects) and the microsomal enzymes which metabolise them:-

(a) All the pharmacologically active morphine-like compounds are metabolised by the liver microsomal enzymes (2,7).

(b) The depression of the pharmacological response in the development of tolerance to a morphine-narcotic and its recovery after the

*$NADPH_2$ in the reduced form of Nicotine Adenine Dinucleotide Phosphate and NADP is the oxidised form. This compound was formerly known as Co-enzyme II or TPN.

withdrawal of the drug is accompanied by a depression and recovery of the N-demethylating activity of the liver microsomal enzymes (7,8,9).

(c) The development of tolerance to a morphine-narcotic is accompanied by the development of tolerance to related drugs and the depression of the liver enzymic activity, similarly, extends to the N-demethylation of related drugs (7,10).

(d) There is a parallel antagonism of the pharmacological activity and the microsomal demethylation of the morphine-narcotics by N-allyl normorphine (Nalorphine) (11).

The work summarised above suggests that if T2636 acts in a similar way to morphine then:-

(1) T2636 should have a pharmacological activity similar to that of morphine and should be N-demethylated by liver microsomal enzymes in vitro.

(2) Chronic administration of T2636 should depress the pharmacological activity and the microsomal N-demethylation of both T2636 and morphine.

(3) Withdrawal of T2636 from tolerant animals should result in the recovery of pharmacological response to the drug and of microsomal N-demethylating activity.

(4) Nalorphine should inhibit the N-demethylation of T2636 by normal liver microsomal preparations.

The experiments described in this paper were designed to test this hypothesis and the results which have been obtained confirmed it.

METHODS

Drugs

Morphine hydrochloride and N-allyl normorphine hydrobromide were made

up in aqueous solution to the appropriate concentrations. The morphine was injected as the hydrochloride but the results have been reported in terms of the free base.

T2636 was supplied as the hydrochloride which is sparingly soluble in water but very soluble in methanol. For the injections a 1 mg/ml solution was made up in 20% aqueous methanol and suitably diluted with water. Since methanol is converted to formaldehyde by liver microsomal preparations, the T2636 solutions for the in vitro studies was made up in alkaline propylene glycol (0.01 m NaOH in 90% aqueous propylene glycol).

Control animals were injected with isotonic saline, 1 ml/kg.

Animals

Adult male alkino rats of the Porton Strain were used in all the experiments. All animals, including the controls, were used only once.

Pharmacological Testing

(1) Tail Clip Test

A large crocodile clip, with polythene covered jaws, was placed about 3 cm from the base of the rat's tail. A positive reaction was a definite attack on the clip within 20 seconds of its application. If the animals did not respond within this time the clip was removed.

(2) Shock Avoidance Test

The apparatus consisted of a box which was divided into two 1 ft x 1 ft compartments, with an aperture in the partition to allow access from one compartment to the other. The base of the cage was made of parallel brass rods through which an electric shock could be applied to the rat through its feet, in either of the compartments. The stimulus used was a 45 volt pulse of 10 msec. duration at a frequency of 50 pulses per second. The test procedure was to place the rat in one compartment and measure the time taken for the rat to find the hole and pass to the unstimulated side of the apparatus. The

maximum period of the stimulation was 50 seconds. Each animal was given a series of six successive stimuli, with a 45 second pause between each stimulus, and the mean time to respond was determined. The animals were trained to respond to the test and most learned very quickly so that after three or four tests, given on successive days, the mean time to respond was less than 5 seconds. This quick response was retained over several weeks, provided the rats were tested in the apparatus every 4-5 days. The results were more consistent if the feet of the rats were bathed in saline just before the test.

Biochemical Methods

A combined microsome plus soluble fraction was prepared from a Potter-Elvehjem homogenate of rat liver and its enzymic activity determined as described in an earlier paper (12). The N-demethylation of morphine and T2636 was determined by measuring the rate of release of formaldehyde which was assayed colorimetrically as diacetyldihydrolutidene (13). The metabolism of T2636 was also measured by estimating the appearance of the secondary amine, N-demethylated T2636, by a modification of the method of Umbreit (14) which is specific for secondary amines in the presence of tertiary and primary amines.

Significance Calculations

The significance of the data was determined by the "Student" T test (15).

RESULTS

The N-demethylation of T2636 by Liver Microsomes

The rates of N-demethylation of T2636 by two preparations of liver microsomes was determined in two different ways: the release of formaldehyde and the formation of secondary amines. The values for the formaldehyde estimations were 1.32 and 1.39 μmole/g/hr and the corresponding data for the secondary amine determinations were 1.28 and 1.31 μmole/g/hr. The two determinations for each preparation were the same, within the limits of the experimental error.

The N-Demethylating Activity of Liver Microsomes from Morphine Tolerant Rats

Rats were made tolerant to morphine by injecting 10 mg/kg (s.c.) daily for 22-24 days. The development of tolerance was followed by measuring the change in analgesic response to the drug, using the Tail Clip Test. It is clear from Table I that the rats had become markedly tolerant to morphine after 21 days. The N-demethylation of both morphine and T2636 was depressed in microsomes prepared from the livers of these rats although the depression of morphine demethylation (62%) was greater than that of T2636 (43%).

This experiment involved the preparation of 13 lots of liver microsomes and the assay of both the morphine and T2636 N-demethylating activity of each preparation. This was impossible to carry out in one day and therefore the microsomal preparations were frozen and stored at -40°C for three to five days before their enzymic activity was determined. This method of storage may result in up to 10% loss of activity (12) but control and experimental preparations were assayed in parallel over the three days and the N-demethylating activity of the preparations stored for 5 days had the same range of values as those stored for 3 days.

The Cross-Tolerance of Morphine and T2636

In this series of experiments the pharmacological response of the rats was measured by the Shock Avoidance Test since it was thought that this technique would demonstrate the development of a small degree of tolerance more clearly than the Tail Clip Test. Six rats were injected with morphine according to the following schedule:- days 1 and 2 - 20 mg/kg; days 3 and 4 - 40 mg/kg; days 5 and 6 - 60 mg/kg; days 7 and 8 - 80 mg/kg and days 9 to 14 - 100 mg/kg. The pharmacological response of the rats to T2636 was determined on Day 11 to morphine on Day 14. The animals were killed and liver microsomes prepared on Day 15. A second group of rats was given a similar series of injections of T2636, rising from 20 to 100 µg/kg.

The pharmacological response of the rats to morphine and T2636 is shown in Table II. The time to respond, measured 24 hours after the last injection, was not affected by pretreatment with either of the drugs for 14 days.

TABLE I

The N-Demethylating Activity of Liver Microsomes from Morphine Tolerant Rats

Treatment	Pharmacological Effect of Morphine*			Rate of Microsomal N-Demethylation (μmole/g liver/hr)		
	No. of Rats Tested	No. Responding Before Injection	No. Responding 2 hr After 10 mg/kg Morphine	No. of Rats	Morphine	T2636
10 mg/kg morphine daily for 21 days	10	10	8	8	2.13 ± 0.51	0.96 ± 0.19
Saline (Controls)	5	5	0	5	5.62 ± 0.89	1.69 ± 0.40
Significance of the Depression of the Microsomal N-Demethylating Activity					$p < 0.01$	$p < 0.01$

*Pharmacological Effect measured by Tail Clip Test: number of animals responding to the clip within 20 seconds of its application.

TABLE II

The Cross-Tolerance of T2636 and Morphine, Demonstrated in the Shock Avoidance Test

Animals	Drug	Dose	Number of Animals	Time to Respond to Shock (Seconds)		Increase in Time to Respond (Sec)	Significance of Increase with Respect to the Controls
				Before Injection	60 min After Injection		
Control	T2636.HCl	25 µg/kg	8	3.40 ± 2.07	35.47 ± 13.26	32.07 ± 13.67	-
	Morphine	50 mg/kg	8	1.06 ± 0.37	43.26 ± 10.30	42.20 ± 10.8	-
T2636 Treated	T2636.HCl	25 µg/kg	6	1.99 ± 1.80	0.78 ± 0.32	-1.21 ± 1.76	$p < 0.01$
	Morphine	50 mg/kg	6	3.88 ± 2.21	1.66 ± 1.29	1.66 ± 1.65	$p < 0.01$
Morphine Treated	T2636.HCl	25 µg/kg	6	1.79 ± 1.75	7.93 ± 4.73	6.14 ± 3.45	$p < 0.01$
	Morphine	50 mg/kg	6	1.01 ± 0.42	1.44 ± 1.73	0.43 ± 1.45	$p < 0.01$

The repeated administration of T2636 completely abolished the effects on the rats of T2636 and of morphine. Similarly,,treatment with morphine completely abolished the effects of the drug itself and markedly reduced those of T2636. Each group of animals had been made tolerant to both T2636 and morphine. Although the dose of morphine was 1,000 times that of T2636 their effects were approximately the same i.e. T2636 is 2,000 times more active than morphine.

The N-demethylation of both T2636 and morphine was depressed in liver microsomal preparations from both T2636 and morphine tolerant rats (Table III), although in the case of the T2636 tolerant animals the depression of T2636 demethylation was barely significant ($0.10 > p > 0.05$). The failure of T2636 to depress its own metabolism may have been due to the relatively small amounts of the drug which were administered since Cochin and Axelrod (16) found that the extent of the depression of microsomal activity in morphine tolerant rats depended upon the dose of morphine being injected at the time the animals were killed and not upon the duration of treatment or the cumulative dose. In order to test this hypothesis five times the dose of T2636, used in the previous experiment, was given to a group of six rats, according to the following schedule:- day 1 - 2 x 100 µg/kg; day 2 - 2 x 150 µg/kg; day 3 - 2 x 200 µg/kg; day 4 - 2 x 250 µg/kg; day 5 - 300 µg/kg; day 6 - 400 µg/kg and day 7 - 500 µg/kg. The animals were killed on the eighth day and liver microsomes prepared. One rat died after the first two injections but the remainder survived the treatment and appeared tolerant to the drug, since the catatonic effect of the last injection disappeared within four hours whereas after the first injection the rats were catatonic for almost six hours. The rates of N-demethylation of both T2636 and morphine by these preparations are shown in Table IV. Both were depressed to a highly significant extent ($p < 0.01$).

The Withdrawal of T2636 from Tolerant Rats

24 rats were given T2636 daily to a final dose of 500 µg/kg, according to the schedule described immediately above except that the maximum dose was repeated on days 8 and 9. On the tenth day the analgesic effect of T2636 (40 µg/kg) was determined using the Tail Clip Test. All the treated

TABLE III

The Microsomal N-Demethylation of T2636 and Morphine in Tolerant Rats

Group of Rats	No. of Rats	Compound	Rate N-Demethylation (μmole/g liver/hr)	Depression of Demethylation (%)	Significance of the Depression of Demethylation
Control	6	Morphine	8.67 ± 2.01	-	-
	6	T2636	2.11 ± 0.69	-	-
T2636 Tolerant	6	Morphine	5.02 ± 1.56	42	$p < 0.01$
	6	T2636	1.45 ± 0.44	29	$0.10 > p > 0.05$
Morphine Tolerant	6	Morphine	1.60 ± 0.83	82	$p < 0.01$
	6	T2636	0.93 ± 0.12	56	$p < 0.01$

TABLE IV

The Microsomal N-Demethylation of T2636 and Morphine in T2636 Treated Rats

Compound	Rate of N-Demethylation (μmole/g liver/hr)				Depression of Demethylation (%)	Significance of the Depression of Demethylation
	No. of Rats	Control Rats	No. of Rats	T2636 Treated Rats		
T2636	6	1.76 ± 0.22	5	0.57 ± 0.24	67	$p < 0.01$
Morphine	6	6.68 ± 1.04	5	1.93 ± 0.62	71	$p < 0.01$

animals were tolerant to T2636 but none of the controls responded to this test (Table V). The control rats were made fully catatonic and lay supine in their cages but the tolerant animals showed signs of excitation and hyperactivity. Half the animals were killed and microsomes prepared from their livers. The N-demethylating activity of the preparations from the tolerant rats was reduced by about 63% towards T2636. T2636 was abruptly withdrawn from the remaining animals and when they were tested 25 days later none of them responded to the Tail Clip after the test dose of T2636. The response of these animals was indistinguishable from that of the controls. The liver microsomes prepared from these rats N-demethylated T2636 at the same rate as preparations from control animals.

In this experiment the microsomal preparations were stored frozen at -40°C for 3 days before assay of their T2636 N-demethylating activity. Experimental and control preparations were assayed in parallel.

The Inhibition of N-Demethylation by Nalorphine

The inhibitory action of nalorphine on the N-demethylation of morphine and of T2636 by liver microsomal preparations from normal rate is illustrated in Figure 1. The demethylation of both compounds is inhibited, although nalorphine is about 1.5 times more potent an inhibitor of morphine demethylation than of T2636 demethylation in equi-molar solutions.

DISCUSSION

The Microsomal Metabolism of T2636

The metabolic experiments reported in this paper were all carried out with the combined microsomal and soluble fractions of the liver cell but it was confirmed that the reaction was brought about by the microsomal enzymes by showing that it occurred when T2636 was incubated with washed microsomes and $NADPH_2$, which was used to replace the NADP and NADP-reducing system of the soluble fraction. There is no doubt that the liver microsomal enzymes N-demethylate T2636 since the amount of formaldehyde released was quantitatively equivalent to the amount of secondary amine appearing in the solution. Thus T2636 and morphine are metabolised in the same way by the same enzyme system.

TABLE V

The Effect of Withdrawal of T2636 from Tolerant Rats

Treatment of Animals	Pharmacological Effects of T2636[x]			Microsomal Demethylation of T2636	
	No. of Rats Tested	No. Responding before Injection	No. Responding 30 min After 40 µg/kg T2636	No. of Rats	µmole/g liver/hr
Increasing doses of T2636 over 10 days. Final Dose 500 µg/kg	20	20	20	10	0.52 ± 0.40*
Saline (controls)	12	12	0	6	1.60 ± 0.38*
Treated with T2636 and then withdrawn from the drug for 25 days.	10	9	0	9	1.82 ± 0.49**
Saline (controls)	6	6	0	5	2.01 ± 0.58**

[x] Pharmacological effect measured by Tail Clip Test: number of animals responding to the clip within 20 seconds of its application.

*Significance of the difference in activity $p < 0.01$

**Significance of the difference in activity $p > 0.9$

A Comparison of T2636 and Morphine

The data presented in this paper have established the following:-

(1) The chronic administration of T2636 to rats results in the development of tolerance, i.e. decreased pharmacological response, to both T2636 and morphine. Similarly, morphine tolerant rats are also tolerant to T2636. However, the two compounds are not completely cross-tolerant since the response to T2636 was not completely abolished in animals which were fully tolerant to morphine.

(2) The development of tolerance to either T2636 or morphine is accompanied by a depression of the N-demethylating activity of the liver microsomal enzymes towards both compounds. The extent of the depression of the microsomal demethylation of T2636 in T2636 tolerance depends upon the dose of T2636 administered, in a similar way to that found in morphine tolerance (16).

(3) On withdrawal of T2636 from tolerant rats the response of the animals to the drug and the microsomal N-demethylating activity return to normal within 25 days. Although the time course of the recovery was not followed in the experiment reported the data fit in with that of Cochin and Economon (9) for morphine withdrawal from tolerant rats. They found that 16 days after withdrawal the pharmacological response was only 40% of the control value, whereas the N-demethylating activity of the microsomes had returned to normal.

(4) Nalorphine inhibits the N-demethylation of both T2636 and morphine by normal liver microsomes: the demethylation of morphine is inhibited about 1.5 times as much as that of T2636, by equi-molar concentrations of nalorphine. A detailed study of the inhibitory action of nalorphine is being made since the nature of the inhibition (competitive or non-competitive)

is of fundamental importance in the assessment of the hypothesis put forward by Beckett et al (6) to explain the mode of action of morphine and by Axelrod (7) to suggest a mechanism for the development of tolerance to the drug. The results of this investigation will be reported in a later paper.

Thus it is apparent that T2636 and morphine have very similar biochemical and pharmacological activities and, as judged by the criteria suggested in the Introduction, it is highly probable that they produce these effects by similar mechanisms. However, T2636 is 500-1,000 times more active pharmacologically than morphine although both compounds are N-demethylated at similar rates by the liver microsomal enzymes.

This dramatic increase in potency produced by the insertion of the ethylene bridge and tertiary alcohol group into the morphine molecules (see Appendix) suggests that the CNS receptor sites of the analgesic drugs may contain a locus which interacts with the polar alcoholic grouping, thus increasing the specificity of the action of T2636. Beckett and Casy (17) have suggested that the analgesic receptor sites contain (a) an anionic site which forms an ionic bond with the basic centre of the drug, (b) a flat surface which binds a flat aromatic structure in the drug by Van der Waals forces and (c) a cavity which can accommodate a projecting hydrocarbon moiety of the drug molecule and thus confer a three-dimensional specificity on the interaction of drug and receptor. If the receptor site is extended to include a fourth locus to interact with the alcoholic grouping then the complete interaction of a drug with the receptor will be very highly specific. Thus T2636, which acts with all four loci in the site is 1,000 times more active than morphine which does not have the tertiary alcohol in the 7 position and hence only reacts with three of the four loci. This postulate is supported by a recent report by Bebbington (18) in which he pointed out that some of the most potent analgesics (nitrobenzamidazole, ethoxybutamoxane and the reversed ester of pethidine) contain a polar grouping equivalent to the tertiary alcohol of T2636 in a similar spatial arrangement to the basic group in the molecule. However, ethoxybutamoxane does not contain a flat aromatic ring in a similar spatial position to that in morphine

although it is a more potent analgesic. Thus the relative importance of the loci or of different combinations of loci, when only three of the four are occupied, in determining pharmacological activity remains to be elucidated. The change of the morphine molecule to T2636 increases its lipid solubility, at physiological pH, and this may be an important factor in facilitating the access of the drug to the receptor e.g. by increasing its penetration of lipoidal membranes. However, these speculations may not be assessed until more information is available about the chemical nature and cytological disposition of the CNS receptor sites of the narcotic analgesics.

The microsomal N-demethylating enzyme system does not have the same degree of specificity as the CNS receptors since T2636 and morphine are metabolised at similar rates. However, the mechanism which depresses the microsomal N-demethylating activity during the development of tolerance has a similar high specificity since the dose of T2636 required to produce a depression in activity is one thousandth that of morphine.

Although the depression of microsomal demethylating activity and the development of tolerance do not occur in strict parallel (16) this work emphasises the similarities of the two processes. Thus a study of the microsomal N-demethylating system may yield results which elucidate the chemical nature of the CNS receptors of the narcotic analgesics.

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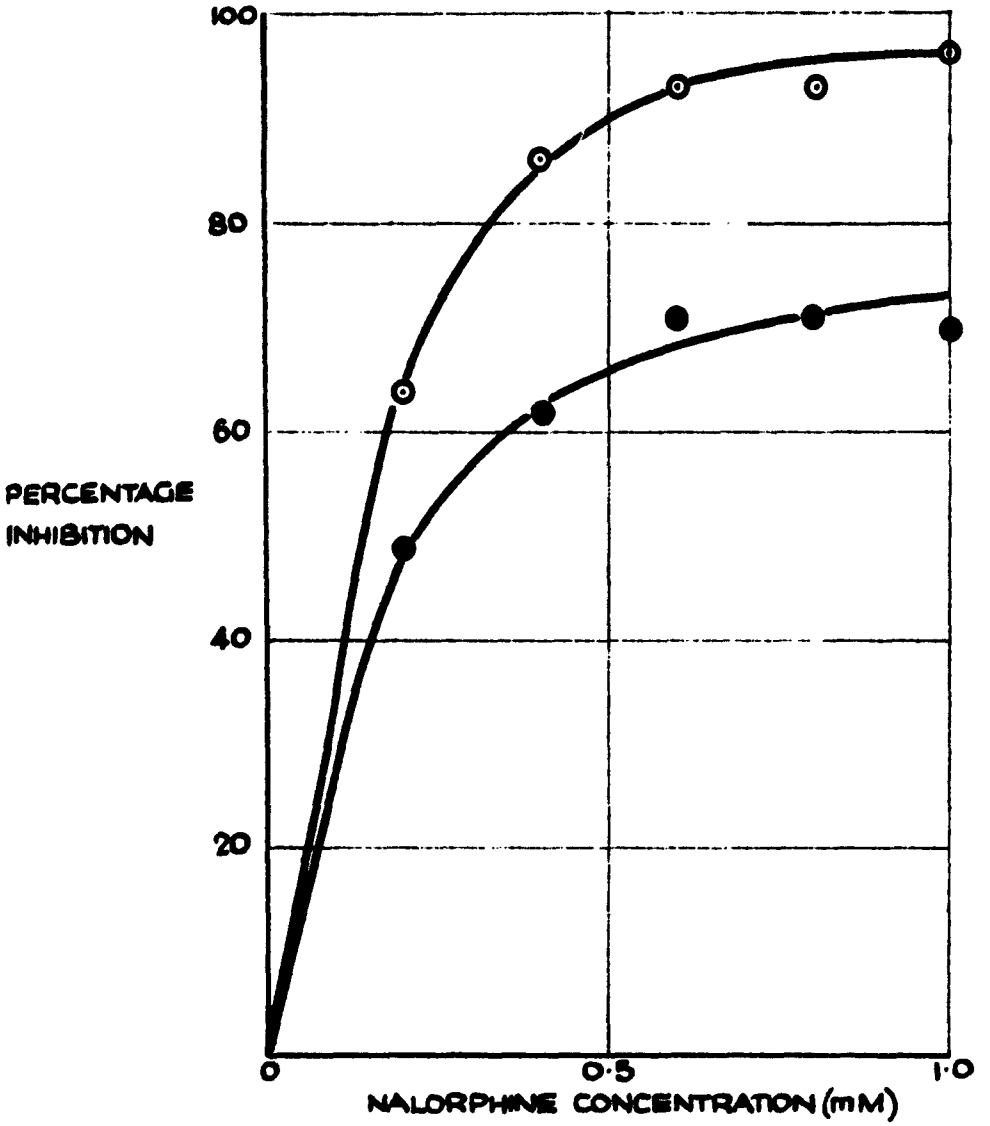


THE CONCENTRATION OF T2636 AND MORPHINE IN THE INCUBATION MIXTURE WAS 0·5 mM. T2636 N-DEMETHYLATION ●——● AND MORPHINE N-DEMETHYLATION O——O

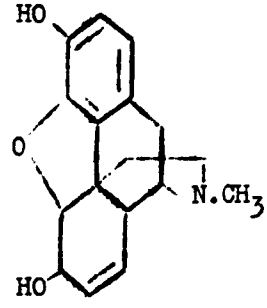
THE INHIBITION OF T2636 AND MORPHINE N-DEMETHYLATION BY NALORPHINE.

FIG.1.

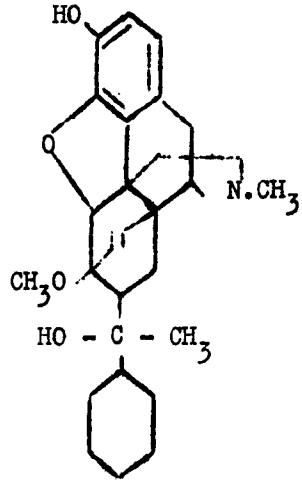
APPENDIX

The Structural Formulae of some of the Compounds mentioned in this Paper

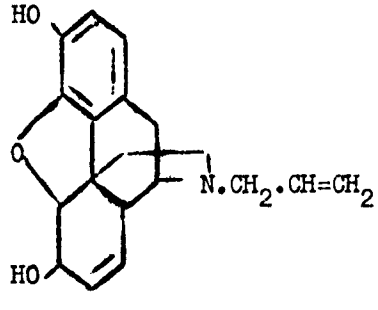
Morphine.



T2636.



N-Allyl Normorphine (Nalorphine).



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