Biochemical Pharmacology Vol. 17, pp. 1099–1105. Pergamon Press. 1968. Printed in Great Britain

# THE PRESENCE IN MICE CHRONICALLY TREATED WITH MORPHINE OF A SUBSTANCE (S) THAT MODIFIES MORPHINE RESPONSES*

F. HUIDOBRO and H. MIRANDA

Department of Pharmacology, Catholic University of Chile, Santiago, Chile

(*Received* 12 *June* 1967, *accepted* 31 *October* 1967)

**Abstract**—A principle that shortens and reduces the intensity of the abstinence syndrome to morphine in white mice was obtained from whole mouse extracts and from urine of mice chronically intoxicated with morphine pellets. Furthermore, a substance that prolongs morphine analgesia was found in extracts from mice implanted with pellets of morphine. At present, it is not known whether both principles are the same.

Procedures followed to obtain the indicated principle(s) are described.

MICE IMPLANTED with pellets of morphine develop tolerance and physical dependence.[1–3] The intensity of the "abstinence syndrome" (AS) induced by nalorphine in these mice decreases as time passes;[1, 4] daily administrations of nalorphine accelerate the rate of this decrement.[5, 6] It is tempting to assume that this effect is caused by some morphine derivative(s) or some other substance(s) produced in the organism, either by the implanted morphine or the injected nalorphine, or by both. However, when extracts obtained from whole mouse homogenates or from urine of implanted mice (receiving nalorphine or not) were run on TLC, no morphine or nalorphine that might be responsible for the AS attenuation was found.[7] On the other hand, some authors,[8–10] have obtained a factor, which alters some effects of morphine, from the blood serum and from the nervous system of animals chronically intoxicated with morphine. Furthermore, the inhibitory action of actinomycin-D on morphine tolerance suggests that animals receiving morphine may produce an antagonist to the alkaloid.[11]

This paper reports the existence in whole mouse homogenates and in urine of white mice implanted with morphine pellets of a substance(s) that reduces the intensity of the AS and increases morphine analgesia.

## METHODS

Adult white mice of both sexes were employed throughout. They were implanted subcutaneously with pellets of morphine (base, weighing 100 mg). The daily amount absorbed from the depot ranged between 100 and 130 mg/kg.† When two or three implantations were performed, allowing a one-week interval between each, the daily

* Supported by a grant from the Gildemeister, Santiago, Chile.

† The quantity absorbed was estimated from the difference in the weight of the pellet before and after the implant.

quantity of morphine absorbed varied between 250 and 300 mg/kg from one group to another.

*Preparation of extracts*

*Whole mouse extracts* (*Series A*). Fifteen groups of 25–100 animals, implanted once or twice with morphine, were employed. They were killed by a blow 2 weeks after the first implant. The skin, tail, limbs and snout were discarded. In this condition, animals were weighed and homogenized in an electric disintegrator with 3 vol. of water.

Step 1. The homogenate was treated with 40% trichloroacetic acid to bring it to pH 1–2, shaken and filtered at vacuum. The filtrate was concentrated at vacuum (72°) to one-tenth of its volume.

Step 2. The filtrate was then dialyzed in cellophane bags against running tap water for 48 hr, and against distilled water for 24 hr. If a precipitate formed, it was discarded by centrifugation.

Step 3. The fraction remaining in the bag was concentrated at vacuum (72°) to a volume equivalent to 3 per cent of the total weight of the animal group. Five vol. of ethanol were added, and the precipitate was discarded by centrifugation.

Step 4. Ethanol was evaporated at vacuum and the aqueous phase was treated with 2 vol. of acetone. The precipitate was eliminated by centrifugation and the liquid fraction was concentrated to dryness at vacuum (72°). The residue was dissolved in a volume of distilled water equivalent to 1·5 per cent of the total weight of the animals. This solution was mechanically shaken for 15 min with 5 vol. of a mixture of petroleum ether and carbon tetrachloride (2:1). It was centrifuged and the organic phase was discarded. This operation was repeated once.

Step 5. The aqueous phase was concentrated to dryness at vacuum (72°). The residue was dissolved in 2 ml of distilled water, filtered through a Sephadex column (2·5 × 50 cm), eluted with distilled water, and collected as several fractions. Each fraction was lyophilyzed, kept at −14°, dissolved in a small quantity of distilled water, and separated into two parts. One of these parts was tried on the AS test (see below). When it proved to modify the AS, the corresponding second part was filtered again through Sephadex, eluted with distilled water, and collected as several fractions. Each fraction was divided into two parts. If one of these parts was active on the AS test, the corresponding second part was filtered again through Sephadex eluted with distilled water, and separated into several fractions, which were lyophilyzed and kept at −14°.

Extracts from similar groups of nonimplanted mice were also prepared.

(*Series B*). In some experiments, mice homogenates were heated (72°) for half an hour instead of adding trichloroacetic acid. HCl was added, keeping the same temperature, until a large amount of precipitate formed, which was eliminated by filtering at vacuum. The liquid phase was concentrated to one-tenth of its volume and dialyzed against distilled water for 48 hr. If a precipitate formed, it was eliminated by centrifugation. The liquid phase was evaporated to dryness (72°) and the residue was not further purified. Extracts from similar groups of nonimplanted mice were also prepared.

*Urine extract.* Five groups of 30–120 mice implanted either two or three times were used. Starting from the sixth day after the first implant, night urine was collected

during the following 10–18 days. Animals had free access to water, but food was supplied only during the day.

The night samples of urine were pooled and stored in a deep freezer. Since the urine was contaminated with feces, it was filtered, and 40% trichloroacetic acid was added to bring it to pH 1–2. The precipitate was discarded by centrifugation. The liquid phase was subjected to the procedure described under whole mouse homogenates, series A. However, before adding ethanol (step 3), the liquid phase was shaken several times with ethyl ether until the ether became colorless, then the ether was discarded. Furthermore, the solution was not shaken with the organic mixture mentioned in step 5. Extracts of urine collected from similar groups of non-implanted mice were also prepared.

*Abstinence syndrome test*

Nalorphine (10 mg/kg, i.p.) injected into mice 7 days after a morphine implantation evokes a characteristic AS 2 or 3 min after the injection. This syndrome has two phases: 1) The first one lasts 20–30 min during which the mice become restless. They jump, walk and run in a disorderly fashion; they leap to the ground or sit and sniff in all directions. Other observed signs are: polypsnea, tear secretion, soft or liquid feces, pilo erection, mictions, priapism, tremors and convulsions that increase if animals are held by the tail, muscular twitches, and writhing and tonic contractions of the back limbs. In a very intense AS, animals may die in a convulsive state. 2) During the second phase, mice remain motionless for 60 min or more and then return gradually to normality.[1]

Therefore, only the first phase was considered in order to quantify the AS in 5 degrees, according to the following symptomatology: first degree, animals are restless and sniff in different directions; second degree, mice occasionally run or jump on the table and sometimes leap to the ground; third degree, animals run, jump and leap to the ground frequently and neurological and neurovegetative signs are weak; fourth degree, intense running, jumping, leaping to the ground and neurological signs are observed; fifth degree, all the indicated signs are very intense and mice may die in convulsions.[3]

Since soft or liquid feces and polypsnea were always observed, independently from the intensity of the AS, they were not considered in the elaboration of this scale. Groups of 4–8 animals were considered as one observed unit in evaluating the AS.

In the experiments here reported, groups of at least 4 implanted mice were employed to test the effects of the whole mouse and urine extracts on the AS intensity. Extracts were dissolved in 1·5 to 2 ml water and each animal received 0·25 ml i.p.; control animals were injected either with 0·25 ml of the extracts from nonimplanted mice or with 0·25 saline. Nalorphine (10 mg/kg, i.p.) was injected ½ hr later to provoke the AS. The intensity of the AS obtained was classified according to the scale of 5 degrees.

*Analgesia test*

Analgesia was tested by the hot plate method.[12] The time that elapsed from the moment in which the animal was placed on the hot plate (56°) until it licked its forelimbs (reaction time) was recorded. The maximal time allowed on the hot plate was 30 sec.

Extracts from mouse homogenates, series B, were dissolved in 2 ml of distilled water and injected into 10 mice; each animal received 0·2 ml i.p. The reaction time on the hot plate was determined 30 min later. Morphine was then injected (10 mg/kg, i.p.) and analgesia was tested ½, 1, 2 and 3 hr after the narcotic. For each extract two control groups were used. One of the groups received 0·2 ml of the solution of an extract from nonimplanted mice and the other group received 0·2 ml of distilled water, ½ hr before the injection of morphine. Results were analyzed statistically.

*Thin-layer chromatography (TLC)*

Urine extracts were tested by TLC, employing silica gel G.[13] The solvents used were methanol–water (1:1) or glacial acetic acid–water (1:2). The developers used were ninhydrin-collidine (detects amino acids and amines),[13] Draggendorff's and iodoplatinate reactives (detect alkaloids)[13] or a 50% sulphuric acid spray, the plate being heated afterward for 30 min at 110°.

## RESULTS

*Abstinence syndrome test.* The injection of whole mouse or urine extracts from implanted animals did not modify the behavior of tolerant mice. The subsequent administration of nalorphine elicited an AS which was always shorter and less intense than in controls; groups treated with extracts from implanted animals showed an AS lasting an average of 10 min and of degree 1 intensity. In control groups an AS of degree 4 intensity lasted 27 min (see Table 1). Implanted mice that received no extract at all had an AS of intensity 3–4, lasting 30 min.

TABLE 1. INTENSITY AND DURATION OF THE ABSTINENCE SYNDROME

| | Expt. No.* | No. of animals | Abstinence syndrome | |
|---|---|---|---|---|
| | | | Intensity† (degrees) | Duration (min) |
| Whole mouse extract | | | | |
| Nonimplanted mice | 123 | 15 | 3 | 30 |
| Implanted mice | 153 | 120 | 1 | 10 |
| | 154 | 67 | 1 | 7 |
| | 155 | 65 | 1 | 10 |
| Urine extract | | | | |
| Non-implanted mice | 157 | 72 | 4 | 27 |
| | 159 | 93 | 4 | 25 |
| Implanted mice | 158 | 59 | 1 | 10 |
| | 160 | 81 | 1 | 15 |
| | 161 | 155 | 1 | 10 |

* Experiments 1–123 were designed to study and improve the extraction procedures.

† For descripion of abstinence syndrome test, see methods.

In some cases, less purified extracts were tried. They were obtained by arresting the extraction procedure before filtration through Sephadex, or even at an earlier step. These extracts depressed the mice before the injection of nalorphine so that they did not run anymore or even stayed motionless. However, the extracts also shortened the AS and decreased its intensity.

Thin-layer chromatography revealed three spots in urine extracts from implanted mice. The first spot was at the application point; the second one had an $R_f$ value of 0·10; and the third had an $R_f$ of 0·80 or 0·95 when methanol or glacial acetic acid was used as a solvent (Table 2). Spots were stained by ninhydrin-collidine reactive. They

TABLE 2. TLC* OF URINE EXTRACTS FROM IMPLANTED MICE

| | $R_f$ values in: | |
|---|---|---|
| | Methanol–water (1:1) | Glacial acetic acid–water (1:2) |
| Urine extract from implanted mice | 0·00 | 0·00 |
| | 0·10 | 0·10 |
| | 0·80 | 0·95 |
| Morphine | 0·30 | 0·65 |
| Normorphine | 0·45 | 0·70 |
| Nalorphine | 0·50 | 0·70 |
| Codeine | 0·40 | 0·60 |

* Ninhydrin-collidine was used as the developer.

were not stained when Draggendorff's or iodoplatinate reactives were applied. Extracts of urine from nonimplanted mice did not produce spots on the TLC plates. Only one of the extracts of whole mouse homogenates from implanted mice was run. Its chromatogram was similar to those obtained with urine extracts.

*Analgesia test.* Whole mouse extracts (series B), obtained either from implanted or nonimplanted animals, did not produce analgesia. However, the analgesic effect of morphine was statistically prolonged when tested in animals that had received, half an hour before, an extract from implanted mice. This effect persisted for 24 hr at least. On the contrary, extracts from nonimplanted mice did not modify analgesia produced by morphine (Fig. 1).

*Physicochemical properties.*—The extract is soluble in water, methanol, and glacial acetic acid; it is almost insoluble in petroleum ether, carbon tetrachloride, ethyl ether, benzene or hexane. When dissolved in glacial acetic acid, it can be precipitated by ethyl ether. It remains active after dialysis (72 hr), incubation with pepsin, 15 min of boiling, or treatment with trichloroacetic acid at 72° for an hour. The biologically active fraction had a maximal light absorption at 250 m$\mu$.

## DISCUSSION

Mice implanted with morphine pellets produce a principle that decreases the intensity and the duration of the AS to morphine induced by nalorphine in tolerant mice. Such a principle was found both in urine and in whole mouse homogenates. It has been impossible to identify the active principle chemically, since the extraction procedure yields a very small quantity of the extract. However, chromatograms suggest that it may be an amine or a polypeptide and not an alkaloid. Apparently it is of a large size, since it does not seem to dialyze. Should it be a polypeptide, it would not be hydrolyzed by pepsin.

Although urine and whole mouse extracts have similar effects on the AS and possess the same physicochemical and chromatographic characteristics, further experiments should be done to determine whether both extracts contain the same principle. The present data do not permit establishment of the dose response curve of the extracts or of the relationship between the amount of implanted morphine and the

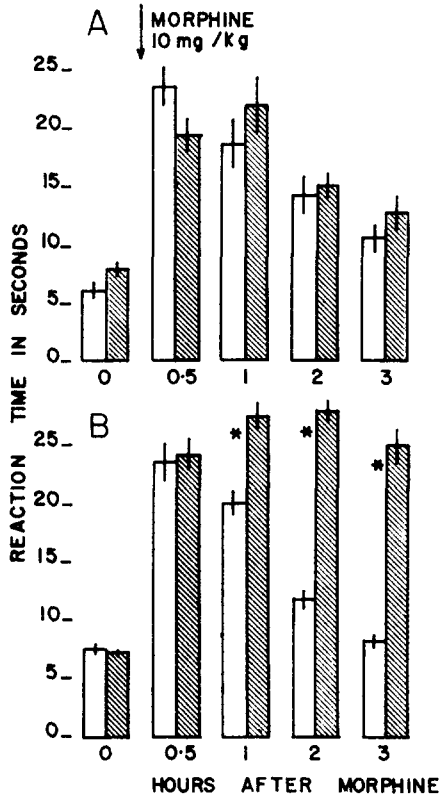


FIG. 1. Ordinates show reaction time to the hot plate (56°).

A. Hatched bars = effect of morphine in mice injected ½ hr before with extracts of normal mice; white bars = effect of morphine in controls.

B. Hatched bars = effect of morphine in mice injected ½ hr before with extracts of mice implanted with pellets of morphine; white bars = effect of morphine in controls.

Vertical lines = S.E.; asterisks = $P < 0{\cdot}01$.

yield of the active principle. Likewise, the possibility that these extracts, when administered in larger doses, might abolish the AS to morphine should be explored.

The factor that prolongs morphine analgesia was not studied further, because when these experiments were carried on, similar results were reported.[9]

It is not known whether the principle that modifies the AS to morphine is the same one that prolongs analgesia; neither can it be excluded that it might be related to the principle that is inhibited by actinomycin-D.[11]

*Acknowledgements*—The authors are grateful for Professor J. Lewin's valuable help and suggestions.

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