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THE METHYLATION OF NORMORPHINE IN RAT BRAIN *IN VIVO**

DORIS H. CLOUET

New York State Research Institute for Neurochemistry and Drug Addiction;
and the
College of Physicians and Surgeons, Columbia University, New York, N.Y., U.S.A.

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Abstract—Labeled methionine was injected into the cisterna magna of the rat, followed 15 min later by normorphine by the same route. When the rats were killed 10 min later, labeled morphine was isolated from the brain.

MORPHINE is N-demethylated to normorphine by mammalian liver *in vitro*. The methylation of the nor-derivative by tissue preparations from rat liver and brain[1, 2] and rabbit lung[3] has been described. In this paper is reported the isolation of labeled morphine from brain after the injection of normorphine and a labeled methyl donor into the cerebrospinal fluid of the rat.

METHODS

Separation of narcotic drugs from brain

The general procedure for the isolation of morphine and normorphine from tissue or an incubation mixture involves organic solvent extraction of the drugs from an alkaline aqueous medium. In experiment I, the procedure of Axelrod[4] was followed except that the organic layer was extracted with 0·1 N hydrochloric acid, and the extraction was repeated in order to decrease the levels of lipids in the extract. In experiment II the procedure of Milthers[5] was followed, again repeating the extraction. The latter procedure proved to be more effective than the first in that the final extract was free of contaminating salts as well as most lipids. The extracts were evaporated to dryness and dissolved in a small volume of alkaline ethanol for application to buffered (pH 7·5 or pH 6·7) filter paper. Chromatography was performed in the solvent system described by Axelrod; the top phase of a tertiary amyl alcohol:n-butyl ether:water mixture in 50:7:43 proportions. Morphine and normorphine were located by ultraviolet absorption and by the iodoplatinate color reaction.

Preparations and measurements of ^{14}C-labeled compounds

In *in vitro* experiments[1, 2] (^{14}C-methyl) S-adenosylmethionine, prepared from ^{14}C-methionine according to the enzymic method of Cantoni,[6] was an effective methyl donor to normorphine. Three different preparations of S-adenosylmethionine prepared in this way were injected into the cerebrospinal fluid of rats and all three were extremely toxic, causing death in less than 2 min at a dose of 0·1 μmole/100 g body-

* Preliminary accounts of some of this work have appeared in *Fed. Proc.* **21**, 326 (1962) and *Acta neurol. scand.*, Suppl. I. **38**, 26 (1962).

weight. The nature of the toxic component was not explored. However, in addition to S-adenosylmethionine, the preparation could have been contaminated with traces of organic solvents used in the isolation procedure, or by Reineckate ion which could be presumed present even after multiple washing since the material had a faint pink color after lyophilization. A precursor, ^{14}C-labeled methionine, was used in the experiments *in vivo* at the dose and specific radioactivity described for each experiment.

Radioactivity of the isolated drugs was measured after separation by paper chromatographic techniques in an automatic chromatogram scanner (Vanguard Instrument Co.) and compared with standard ^{14}C-methionine spots on paper measured at the same instrument settings.[2]

Production and measurement of the analgesic response

Because normorphine is an active analgesic agent with lethal effects at relatively low doses, it was necessary to assess the response of rats to the intracisternal administration of the drug.

Male rats of the Sherman strain, weighing 80 to 120 g, were used in these experiments. For injection into the cerebrospinal fluid, normorphine hydrochloride was neutralized before adjusting volume so that the dose for a 100-g rat was contained in 0·025 ml. The rats were lightly anesthetized with ether prior to the injection of normorphine into the cisterna magna. Recovery from ether anesthesia occurred in less than 30 sec. Analgesia was measured by the hot-plate method[7] at 5-min intervals beginning either 2·5 or 5 min after the injection. An approximate LD_{50} of 1·09 mg normorphine base/kg was calculated from the mortality ratios at doses from 0·5 to 2·0 mg/kg. Since a maximal level of normorphine in the brain was desirable, doses close to the LD_{50} were adopted. In this range (1·0 to 1·4 mg/kg) analgesia was found at the 2·5- and 5-min tests and lasted at least 2 hr.

An attempt to determine an effective dose of meperidine introduced by the same route was not successful. Analgesia was not produced in the range of 0·1 to 2·0 mg/kg despite the fact that doses above 1·5 mg/kg were uniformly lethal in less than 2 min after injection of meperidine into the cisterna magna. In experiment II, therefore, meperidine was injected subcutaneously to produce a level of analgesia equivalent when measured by the hot-plate test to that produced by the intracisternal injection of 1 mg normorphine/kg.

RESULTS

Since the choice of a suitable time period for the administration of both normorphine and labeled methionine would control to a large degree the possibility of demonstrating methylation *in vivo*, we have considered the timing for each component separately. For normorphine, maximal brain levels were presumed to be related to the analgesic response so that the optimal time to detect metabolic reactions of the drug would be related to the initiation of analgesia in the rat (2·5 min after the injection of normorphine).

The time course of appearance of the label in the free amino acid pool of the rat brain (i.e. trichloroacetic acid-soluble fraction) after the injection of a labeled amino acid into the cisterna magna has been described for ^{35}S-methionine[8] and ^{14}C-lysine.[9] For both amino acids, peaks of radioactivity were found in the nonprotein at times between 10 and 30 min. Similar half-life times in brain for other amino acids have been

found after the subarachnoidal injection of the labeled compound.[10] The presence of a methyl-activating system in rat brain was presumed from evidence indicating methylation of normorphine,[1] histamine,[11] and choline[12] by brain tissue preparations *in vitro*. Thus, the maximal level of activated ^{14}C-methyl groups may be expected at some time period later than 10 min after the injection of (^{14}C-methyl) methionine, the exact time related to the availability of methionine to the activating system.

In preliminary experiments the timing was varied within the limits suggested by these considerations. The length of time between the injection of methionine and normorphine was varied from zero (injection in the same solution) to 30 min, and the time for termination of the experiment varied from 2·5 min after the injection of normorphine to 30 min after the injection. In only one of these preliminary experiments was there a suggestion of methylation. In this experiment a 136-g rat was injected with 0·53 μmole (2·5 μc) of methionine at zero time, 0·136 mg of normorphine 15 min later, and was killed 25 min after the first injection; radioactivity was found at the R_f of morphine in a chromatogram of an organic solvent extract prepared from the brain. This time schedule was adopted for the later experiments.

Experiment I

Six rats were injected with (^{14}C-methyl)-methionine at a dose of 0·5 μmole (2·5 μc) in 0·025 ml/100 g rat; 15 min later 0·136 mg normorphine base (0·5 μmole)/100 g rat was injected into the cisterna magna of four of the six rats. Maximal analgesic response (no reaction to the heat stimulus in 20 sec) was present at 5 min for all four rats. Twenty-five min after the injection of labeled methionine all six rats were killed by decapitation. The brains were removed, rinsed in ice-cold water and homogenized in 10% trichloroacetic acid. After centrifugation, 0·5 μmole morphine was added to each acid-soluble fraction, and the sample was subjected to the procedures for isolation and chromatography described in the preceding section.

As may be seen in Table 1, radioactivity at the R_f of morphine was obtained in the preparation from each of the four rats injected with normorphine, and none in the control rats injected only with labeled methionine.

TABLE 1. METHYLATION *in vivo*; EXPERIMENT I

Rat No.	Body weight (g)	Injection into cisterna magna: Zero time Methionine (μmoles)	Injection into cisterna magna: 15 min Normorphine (μmoles)	Hot-plate response (sec): Pre-inj.	Hot-plate response (sec): 20 min	Hot-plate response (sec): 25 min	Chromatography pH 7·5 paper: (Rf)	Chromatography pH 7·5 paper: (cpm)
1	88	0·44	0·44	5	>20	>20	0·75	7
2	80	0·40	0·40	2	>20	>20	0·71	6
3	85	0·43	0·43	2	>20	>20	0·74	8
4	91	0·46	0·46	7	>20	>20	0·72	10
5	89	0·45	0	3	4		0·71	0
6	80	0·40	0	3	3		0·70	0

Experiment II

The experiment was repeated with an additional control group of two rats in which analgesia was induced by meperidine in order to eliminate the possibility that the labeled product was formed from methionine during analgesia without the presence of

normorphine. (^{14}C-methyl)-methionine was injected into the cisterna magna of six rats at a dose of 0·35 μmole (0·35 μc)/100 g body weight at zero time. Fifteen min later, 0·37 μmole of normorphine/100 g was injected into the cisterna magna of two of the rats and 2 mg/100 g rat of meperidine subcutaneously into two others. All four rats had a maximal analgesic response 5 min later. All six rats plus two untreated control animals were killed 25 min after the injection of methionine. The brains were removed, rinsed in ice-cold water, and homogenized in saline. Concentrated hydrochloric acid was added to make a 1 N solution and the precipitated protein was removed by centrifugation. To each acid-soluble fraction 0·5 μmole of morphine was added, and the isolation procedure of Milthers was followed by chromatography on paper buffered at pH 6·7 by 0·5 M phosphate buffer in the solvent system described above.

TABLE 2. METHYLATION *in vivo*; EXPERIMENT II

Rat No.	Body weight (g)	Injections: Zero time Methionine (μmoles)	Injections: 15 min Normorphine (μmoles)	Injections: 15 min Meperidine (mg subcu.)	Hot-plate response (sec): Pre-inj.	Hot-plate response (sec): 20 min	Hot-plate response (sec): 25 min	Chromatography pH 6·7: (Rf)	Chromatography pH 6·7: (cpm)
1	87	0	0	0	6			0·42	0
2	81	0	0	0	3			0·47	0
3	85	0·30	0	0	2			0·50	0
4	80	0·28	0	0	4			0·44	0
5	111	0·39	0	2·22	5	>20	>20	0·44	0
6	112	0·39	0	2·24	3	>20	>20	0·48	0
7	99	0·35	0·37	0	3	>20	>20	0·48	20
8	105	0·37	0·39	0	2	>20	>20	0·43	11

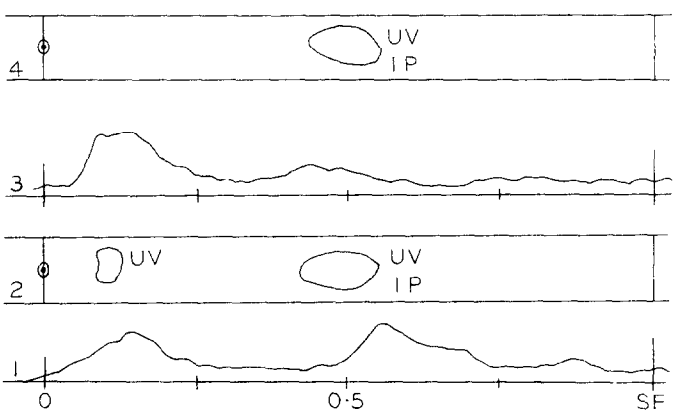

FIG. 1. Chromatographic strip and sutoscanner record from rats 6 and 7 of experiment II. Lines 1 and 3 are autoscanner records of 7 and 6, respectively, and lines 2 and 4 are chromatograms of 7 and 6 respectively. O = origin, SF = solvent front, IP = positive iodoplatinate reaction, UV = ultraviolet absorption. The right ordinate mark on the autoscanner records equals 10 cpm at the instrument settings used.

The results of this experiment (Table 2) were the same as those of the first, with additional information from the control anesthetized group. In these two rats, analgesia produced by the subcutaneous injection of 20 mg meperidine/kg did not result in the appearance of label at the R_f of morphine. Figure 1 shows the chromatographic

strip and tracing from rats number 6 and 7 of Table 2. The peak of radioactivity and iodoplatinate color reaction at R_f 0·48 coincided with those of morphine. Other areas of radioactivity and ultraviolet absorption at an R_f of 0·10 may be ascribed to trace methionine and normorphine respectively.

When carrier morphine was not added to the acid filtrate, labeled morphine was not recovered consistently.

DISCUSSION

The basis of the identification of the labeled product as morphine is the behavior of the labeled compound during solvent extraction and chromatography. The lack of a radioactive spot at the R_f of morphine in samples from control rats in which conditions were duplicated except for the injection of normorphine, eliminates the possibility that the labeled spot is a lipid with the same solubility and chromatographic characterstics as morphine. The transfer of the labeled group to the nitrogen of normorphine is indicated by the knowledge that a possible transfer elsewhere on the molecule would result in a compound such as norcodeine with a different R_f value. In our studies on the methylation of normorphine *in vitro* by liver and brain preparations with S-adenosylmethionine as the methyl donor, the presence of several additional spots of radioactivity made it necessary to use other solvent systems in two-dimensional chromatography to resolve and identify the spots. In these experiments *in vitro*, the radioactive spot at the R_f of morphine in the solvent system used here moved to the R_f of morphine in two other solvent systems.[2]

An approximation of the amount of labeled morphine isolated from the brain in experiment II may be made on the basis of the ^{14}C-standardization of the autoscanner as 1 count per min equivalent to $1{\cdot}28 \times 10^{-5}$ μc of radiocarbon at the settings used to count the paper strips. In rats 7 and 8, 2·56 and $1{\cdot}41 \times 10^{-4}$ μmoles morphine were formed. Thus, out of 3800×10^{-4} μmoles normorphine injected, only 2×10^{-4} μmoles morphine were recovered—a conversion of 0·053 per cent. However, the amount of morphine calculated in this way is a minimal value since it was assumed that there had been no dilution of the labeled methyl group. It is likely, in view of the well-known effects of pool dilution of amino acids in brain,[13] that both unlabeled methionine and other activated methyl groups contributed to the pool of methyl donors.

An alternative mechanism of transport of normorphine from the cerebrospinal fluid to the systemic circulation and methylation in the liver or other organ, with subsequent transport of morphine to the brain, cannot be excluded in these experiments. It seems to be unnecessary to postulate such a mechanism since N-methylation has been demonstrated *in vitro* in brain preparations, although final proof would rest on the demonstration of methylation in eviscerated animals.

Eviscerated rats were used in recent experiments in which normorphine was recovered from the brain after the injection of morphine.[14] Such demonstrations of methyl transfer during the period of pharmacological activity may eventually offer evidence concerning the various hypotheses relating the analgesia produced by morphine, and tolerance to it, to N-demethylation of the drug.[15, 16]

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REFERENCES

1. D. H. Clouet, *Life Sci.* **1**, 31 (1962).
2. D. H. Clouet, M. Ratner and M. Kurzman, *Biochem. Pharmacol.* **12**, 957 (1963).

3. J. AXELROD, *Life Sci.* **1**, 29 (1962).
4. J. AXELROD, *Science* **124**, 263 (1956).
5. K. MILTHERS, *Acta pharmacol. (Kbh.)* **18**, 199 (1961).
6. G. L. CANTONI in *Methods in Enzymology*, vol. 3, p. 600. Academic Press, New York (1957).
7. N. B. EDDY and D. LEIMBACH, *J. Pharmacol. exp. Ther.* **107**, 385 (1953).
8. D. H. CLOUET and D. RICHTER, *J. Neurochem.* **3**, 219 (1959).
9. D. H. CLOUET and H. WAELSCH, *J. Neurochem.* **10**, 51 (1963).
10. A. LAJTHA and J. TOTH, *J. Neurochem.* **9**, 199 (1962).
11. D. D. BROWN, J. AXELROD and R. TOMCHICK, *Nature, Lond.* **183**, 680 (1959).
12. J. BREMER and D. M. GREENBERG, *Biochim. biophys. Acta* **46**, 205 (1961).
13. H. WAELSCH, in *Amino Acid Pools*, J. T. HOLDEN, Ed., p. 722. Elsevier, Amsterdam (1962).
14. K. MILTHERS, *Nature, Lond.* **195**, 607 (1962).
15. J. AXELROD, *J. Pharmacol. exp. Ther.* **117**, 322 (1956).
16. A. H. BECKETT, A. F. CASY and N. J. HARPER, *J. Pharmacol.* **8**, 874 (1956).