11. Cardoso, S. S., Camargo, A. C. M., Costa, S. R., ibid., Abst. 65.

12. Cardoso, S. S., Camargo, A. C. M., Ferrreira, A. L., submitted for publication.

13. Higgins, G. M., Anderson, R. M., A. M. A. Arch. Path., 1931, v12, 186.

14. Lison, L., Histochimie et Cytochimie Animales. Principes et Méthodes. 3rd Ed. Gauthiers-Villars, Paris, 1960.

15. Harkness, R. D., Brit. Med. Bull., 1957, v13, 87.

16. Cameron, C. B., ibid., 1957, v13, 119.

Received November 23, 1966. P.S.E.B.M., 1967, v124.

Effect of Nalorphine and Levallorphan on Brain Concentrations of Levorphanol in the Dog.* (31947)

KIRK D. WUEPPER,† S. Y. YEH,‡ AND L. A. WOODS‡

Departments of Pharmacology, University of Michigan Medical School, Ann Arbor and University of Iowa College of Medicine, Iowa City

The remarkable antagonism of narcotic analgesic agents by nalorphine has stimulated recent efforts toward elucidation of the mechanism(s) of narcotic drug action and particularly the afore-mentioned antagonism. One possible explanation of the latter is alteration of CNS distribution of narcotic drug by nalorphine. Since the absolute amount of most narcotic agents present in the CNS is small after pharmacologic doses, studies on alteration of selective CNS distribution depend upon accurate and highly-sensitive methods of drug estimation. The latter requirement has now been met with the availability of radioactive-labeled narcotic drugs. One such radioactive synthetic analgesic, N-C^{14}-methyl levorphanol, has been prepared(1) and was utilized in the present study. This communication reports data obtained in experiments showing the effect of nalorphine or levallorphan on the selective distribution of radioactive-labeled levorphanol in the CNS of the dog after subcutaneous injection.

Methods. 1) Synthesis of C^{14}-methyl labeled levorphanol. Early experiments (Univ. of Michigan, 1959-60) utilized labeled drug prepared by the method of N-formylation and catalytic reduction described previously(1). The more recent studies (Univ. of Iowa, 1964) were done with C^{14}-labeled levorphanol prepared by a modification of the synthesis of N-C^{14}-methyl labeled morphine(2).

A mixture of 120 mg (0.2 mM) of levo-3-hydroxymorphinan, 5 ml of absolute-methanol, 15 mg of C^{14}-paraformaldehyde (0.5 mM, approximately 2 mc/mM) and 0.5 ml (13 mM) 97% formic acid were placed in a 15 ml flask and heated to reflux for 6 hours at 100°C (bath temperature). Then 15 mg of non-labeled paraformaldehyde was added and the mixture was again heated to reflux for another 6 hours. The alcohol and excess formic acid were removed under nitrogen and reduced pressure and the white residue was dissolved in 3 ml of 0.25 N HCl. The crude product was precipitated from the acid by addition of aq. NH_4OH. The mixture was centrifuged and the precipitate washed twice with 1 ml of ice-cold water and dried *in vacuo* in an Abderhalden apparatus for 2 hours at 56°C. The crude product weighed 120 mg (88% from the morphinan reactant) and contained about 10% impurity. A solution of the above impure product in 3 ml of absolute methanol was passed through a column (400 × 9 mm) containing 5 g of neutral alumina and eluted with 80 ml of absolute methanol. After the solvent was removed under nitrogen and reduced pressure in the absence of light, the residue was dissolved in 3 ml of 0.25 N HCl. Insoluble material was removed by passing the solution through filter paper and

---

*These studies were supported in part by NIH research grants NB-625 and NB-02928.

†Present address: Division of Dermatology, Dept. of Medicine, San Franciso Medical Center, Univ. of California.

‡Present address: Dept. of Pharmacology, University of Iowa College of Med., Iowa City.

the base was precipitated by the addition of aq. NH_4OH. The mixture was centrifuged and the precipitate washed twice with 1 ml of ice-cold water and dried *in vacuo* for 4 hours at 56°C. The dried residue was dissolved in 0.5 ml of tertiary butyl alcohol and crystallization of the alcohol complex was induced by seeding and the excess alcohol removed *in vacuo* giving a yield of the complex of 60 mg.

The tertiary butyl alcohol complex and 31 mg of d-tartaric acid were dissolved with warming in 0.75 ml of distilled H_2O. The solution was slowly cooled to room temperature, seeded and allowed to stand for 24 hours. The crystals of the C^{14}-labeled levorphanol d-tartrate were removed by centrifugation, washed with a minimal amount of ice-cold water and dried *in vacuo* for 4 hours at temperature 56°C. The yield was 25 mg of a product shown to have identical R_f values with authentic non-labeled levorphanol on chromatography. Radioactivity was demonstrated in only one spot, that of levorphanol, and the specific activity of the tartrate salt was about 1.6 μc/mg.

2) Estimation of C^{14}-labeled levorphanol. The procedure utilized was that described by Woods *et al*(1) except that 10 ml aliquots of the final $EtCl_2$ was evaporated in a scintillation counting vial on a Fisher slide warmer at 55°C in a fume hood. The dried residue in each vial was dissolved by swirling with 0.8 ml of *n*-amyl alcohol and then mixed with 10 ml of phosphor toluene solution§ and each sample counted in a liquid scintillation spectrometer for sufficient time to give a counting error of less than 2%. All samples were analyzed in duplicate. One-third of the experiments were performed without the "reextraction step" described previously(1) since the procedure was specific (shown by paper chromatography) without the extra manipulation.

Samples (see section on animal experiments for origin) of brain tissue were thawed, weighed accurately on a Mettler balance and homogenized (Teflon pestle-pyrex tube cooled in an ice bath) either in 0.5 N HCl or 0.007 M NH_4OH in a total volume of 10 ml. The maximum concentration of tissue homogenate was 10%. After the tissue was homogenized, 4 ml aliquots were transferred to glass stopper centrifuge tubes and the HCl homogenates neutralized with a small volume of conc. KOH (*ca* 40%) or NH_4OH and then carried through the procedure described previously.

Compared with aqueous solutions, recovery of known quantities of C^{14}-labeled levorphanol added to brain tissue in 10% homogenates was about 99 ± 5% (SD) and from plasma was 101 ± 4% (SD).

When samples of CNS tissue amounted to less than 50-100 mg, there was appreciable variation from animal to animal as, for example, the hypothalamus. The variation resulted from the low total radioactive counts because of the small quantity of tissue, thus greater experimental error, although it was apparent that on the average the actual concentrations per unit weight of tissue were significant. Such difficulties were not encountered for tissues as the cortex, cerebellum, or medulla.

3) Animal experiments. Dogs weighing 7-11 kg were injected subcutaneously with aqueous solutions of each drug in the dorsum several inches from the midline. When both the agonist and the antagonist were administered, each was given *via* a separate syringe into a different injection area. Drug solutions of 0.5% (calculated as free base) were used and when both antagonist and agonist were administered, the injections were made within a few seconds of each other. The doses were 2 mg/kg for each drug.

The dogs were sacrificed at the desired time by rapid intravenous injections of 40 mg/kg of thiopental sodium followed by immediate and extensive incision of the right ventricle. Two-thirds of the animals (Univ. of Michigan) were perfused immediately with physiological saline solution into the aorta under a pressure of 80-100 mm Hg, the total perfusion volume being 1,000 ± 200 ml. The other third of the animals (Univ. of Iowa) were not perfused and the entrapped blood in brain tissue was estimated(3). The actual concentrations in the central nervous system were corrected by

---

§ A solution of 3 g of 2,5-diphenyloxazole (PPO) and 100 mg of 1,4-bis-2-(5-phenyloxazolyl)-benzene (POP OP) dissolved in 1 liter of analytical grade toluene.

TABLE I. Typical Results from Experiments* on Effect of Administration of Nalorphine or Levallorphan upon Selective Distribution of N-C^{14}-Methyl Levorphanol in Dog Brain.

	Conc. of C^{14}-levorphanol, ng/g or ml		
Tissue or fluid	Control† C^{14}-levorphanol	C^{14}-levorphanol with nalorphine‡	C^{14}-levorphanol with levallorphan§
Frontal gray	1860	1260	1300
" white	1160	590	550
Temporal gray	1870	1350	1200
" white	1280	850	750
Parietal gray	2330	1420	1400
" white	1420	610	700
Occipital gray	1920	1450	1450
" white	1250	650	600
Mean cort. gray	2000	1370	1340
" cort. white	1280	680	650
Thalamus	1510	1110	1300
Hypothalamus	4170	2470	1100
Corpus callosum	990	610	550
Pons	1300	960	950
Medulla	1440	820	950
Corpora quad.	1810	990	1400
Plasma	256	277	220

* Both the agonist and antagonist were given simultaneously in 2 separate subcutaneous injections of 2 mg/kg, each. The dogs were sacrificed 30 min after administration of the drug(s).

† One of 5 animals. ‡ One of 4 animals. § One of 2 animals.

the drug in the entrapped blood. The brain was removed, cooled quickly below 0°C, and dissected into individual samples as desired. The samples were wrapped in aluminum foil, labeled, frozen quickly on solid CO_2 and stored in a freezer until analyses were performed. Plasma samples were obtained at selected intervals, including one at time of sacrifice, and analyzed for concentration of C^{14}-labeled levorphanol alone; 4 dogs received both labeled levorphanol and non-labeled nalorphine, and 2 dogs were injected with levallorphan and labeled levorphanol. Each animal was sacrificed 30 minutes after injection of drug(s).

Results and discussion. Data obtained in a typical experiment are presented in Table I; one dog each for control (only radioactive-labeled levorphanol), nalorphine-levorphanol, and levallorphan-levorphanol. The experimental variation is typical, being relatively little for tissues permitting large sampling and greater for tissues available in small amounts.

Since 5 dogs served as control animals and 4 dogs in the levorphanol-nalorphine group, data were ample for statistical analysis of the results. Statistical evaluation is given in Table II for cortical gray, cortical white, medullary, and plasma samples at time of sacrifice. Each of these biological samples was available in adequate quantity to provide very reliable quantitative analysis, although many of the other brain samples showed similar quantitative differences. An obvious conclusion is that, under the conditions of the experiments, nalorphine reduces significantly the CNS concentration of levorphanol although nalorphine has no significant effect on the plasma concentrations of levorphanol. The experimental data were accumulated in two different laboratories by two groups of investigators using samples of radioactive-labeled drugs synthesized by two different procedures. The results from the two laboratories were substantially the same.

Although only 2 animals were used in studying the effect of levallorphan upon brain concentrations of levorphanol, the results of the two experiments checked very well with each other and fell in the range of the results shown for the nalorphine-levorphanol experiments noted in Table II. Thus the le-

TABLE II. Statistical Analysis of Effect of Nalorphine upon Selective Distribution of N-C^{14}-Methyl Levorphanol in Dog Brain.*

	Conc. of C^{14}-levorphanol, ng/g or ml					
	C^{14}-levorphanol (5 dogs) †		C^{14}-levorphanol plus nalorphine (4 dogs) ‡		Statistical values	
Tissue or fluid	Mean	Range	Mean	Range	t value	P value
Cortical gray	2480	1760–3030	1350	1150–1630	3.75	$<.01$
Cortical white	1250	830–1390	760	680– 900	3.63	$<.01$
Medulla	1680	1140–2100	990	820–1300	3.11	$<.01$
Plasma	330	256– 560§	290	277– 325	.76	$>.05$

* Both the agonist and antagonist were given simultaneously in 2 separate subcutaneous injections of 2 mg/kg, each. The dogs were sacrificed 30 min after administration of the drug(s).

† 3 dogs were sacrificed in experiments performed at Univ. of Michigan, 1959 and 1960. Two animals were studied at Univ. of Iowa, 1964, using C^{14}-levorphanol prepared by a new procedure.

‡ 2 dogs were studied in each of the laboratories described in footnote "†" at the indicated time.

§ 30 min plasma values of additional control animals were included to give a total of 8. 7 values were in the range 256-378; one value was 560.

vorphanol-concentration lowering action is not peculiar to nalorphine but is characteristic of "narcotic-antagonist" drugs.

There are several possible explanations for the effect of narcotic antagonists on brain levels of levorphanol in the dog. One is that the antagonist alters the rate of absorption and/or detoxication of the levorphanol. However, this suggestion is unlikely since the plasma levels of free levorphanol were not significantly different for the two groups of animals: levorphanol only and levorphanol-antagonist treated. If purely passive physical-chemical relationships existed, the brain plasma ratios should be identical in the two groups of experimental animals which was not the case.

Another possibility is a difference in pharmacological effects of levorphanol in agonist-treated *vs* agonist-antagonist-treated dogs. There was no overt evidence for such a difference since only one of the total of 11 animals showed significant depression of the CNS, the other 10 animals being active and alert although qualitatively showing analgesic effects in the agonist-treated group.

One is left with the explanation that the levorphanol-antagonist effects result from an action at the local level, perhaps at the "blood-brain barrier" or by alterations in intra-cerebral vascularity. Conceivably there could be an interference in passive membrane penetration at the molecular level or an alteration of an active metabolic transport mechanism.

Before becoming enthusiastic about the results of the above levorphanol-antagonistic studies as an explanation of the narcotic analgesic-antagonist phenomena, one must remember that nalorphine tends to *increase* the CNS concentrations of C^{14}-labeled morphine in the dog(4). However, the time-concentration pattern of morphine shows a relative plateau during the 30-minute to 8-hour period after injection(5,6), whereas levorphanol penetrates rapidly to reach maximum concentrations at about 30 minutes and then equally rapidly disappears(7) from the CNS of the dog.

Summary. Simultaneous administration of 2 mg/kg of either nalorphine or levallorphan with C^{14}-labeled levorphanol, 2 mg/kg, in the dog results in a marked diminution of brain concentrations of levorphanol as compared with control. On the other hand, the antagonist drugs did not alter significantly the plasma levels of the levorphanol. The most satisfactory explanation at present is a local brain effect, intra-cerebral vascular, physical-chemical at the molecular level reducing passive membrane penetration, or alteration of an active metabolic process. The phenomenon is not characteristic of all narcotic analgesics, the nalorphine-morphine result being a noted exception.

---

1. Woods, L. A., Mellett, L. B., Andersen, K. S., J. Pharmacol. Exp. Therap., 1958, v124, 1.

2. Andersen, K. S., Woods, L. A., J. Org. Chem., 1959, v24, 274.

3. Meijer, A. E. F. H., Clin. Chim. Acta, 1962, v7, 638.
4. Mule, S. T., Woods, L. A., Mellett, L. B., J. Pharmacol. Exp. Therap., 1962, v136, 242.
5. Mule, S. T., Woods, L. A., ibid., 1962, v136, 232.
6. Woods, L. A., ibid., 1957, v120, 58.
7. Laird, R. D., Woods, L. A., Abstracts, Fall meeting, Am. Soc. Pharm. Exp. Therap., 1958, 21.

Received November 28, 1966. P.S.E.B.M., 1967, v124.

Inapparent Herpes Simplex Virus Infection in Inoculated Rabbits. (31948)

WARREN K. ASHE AND ANTHONY A. RIZZO
(Introduced by S. E. Mergenhagen)
U. S. Department of HEW, Public Health Service, National Institutes of Health, National Institute of Dental Research, Bethesda, Md.

A recent study of experimentally induced herpes simplex virus (HSV) ulcerations in the oral mucosa of rabbits showed that the lesion not only spread laterally along the mucosal surface but also extended downward for a considerable distance into the ducts of the minor salivary glands located well below the surface epithelium(1). Histologic signs of the infection persisted in these ducts several days longer than in lesions on the mucosal surface. This observation suggested that an inapparent herpetic infection of short duration was produced in the gland ducts. Since these ducts are only approximately 1 mm in length, the susceptible epithelial cells necessary for HSV replication were rapidly destroyed thus limiting the duration of the infection in this site. As a result, it was conceivable that an inapparent HSV infection involving the longer ducts of a major salivary gland, where more tissue would be available for viral replication, would persist for a longer period of time. Therefore, the present studies were undertaken to investigate this possibility using cultural, serological and histological methods. A local infection involving the ducts of the submaxillary glands was induced by direct inoculation of HSV into the duct orifice tissue. In view of reports that HSV is readily cultivable from the saliva of rabbits with active herpetic infections(1,2,3), tests were performed on specimens of saliva taken after gross oral ulcerations had healed. Similarly, serum specimens were assayed for HSV neutralizing antibody and ductal specimens were examined for histologic signs of HSV infection.

Materials and methods. Solution and media. Dulbecco's(4) phosphate buffered saline (PBS) was prepared in glass-distilled water and sterilized by Seitz filtration. Trypsin solution (0.25%), lactalbumin hydrolysate medium(5), and Eagle's medium(6) were prepared as described in a previous report(7). Antibiotics (penicillin G, 100 units/ml; streptomycin, 100 ug/ml; tetracycline, 10 μg/ml; nystatin, 100 units/ml) and sera as required, were added to these media at the time they were used.

Cell cultures. Kidneys of 8-10-week-old New Zealand white rabbits (NIH strain) were trypsinized(7) and the dispersed cells were suspended in lactalbumin hydrolysate medium supplemented with 8% inactivated calf serum in a ratio of 1 ml of cells per 125 volumes of medium, yielding an average viable cell count of 5.4×10^5 cells per ml. Portions (0.9 ml) of this suspension were dispersed into disposable(8) 16 × 150 mm roller tubes and incubated in a stationary position at 37°C for 4 to 5 days. The medium was changed (0.9 ml) and the cultures were inoculated when a confluent cell sheet had formed. A substrain of the S-3 clone(9) of HeLa cells was maintained as stock cultures in Eagle's medium supplemented with 10% inactivated horse serum. Tube cultures were prepared using 0.5 ml of cell suspension containing approximately 10^5 viable cells. Following 24-hr incubation at 37°C, the medium was changed and the cultures were inoculated 1 to 2 days later.