[Chem. Pharm. Bull. 24(5) 901—906 (1976)]

UDC 547.94.04.09 : 615.212.7.011.5.076.9

Chemical Reactivity of Morphine and Morphine-6-conjugates and Their Binding to Rat Brain

Hidetoshi Yoshimura,[1a] Reiko Natsuki,[1b] Seiu Îda,[1a,c] and Kazuta Oguri[1a]

Faculty of Pharmaceutical Sciences, Kyushu University[1a] *and Faculty of Pharmaceutical Sciences, Nagasaki University*[1b]

(Received August 1, 1975)

By substitution reaction with nucleophilic reagents such as lithium chloride and piperidine, morphine-6-conjugates were found to be convertible easily to 6-chloro-6-deoxymorphine and 8-piperidino-6-deoxy-Δ^6-morphine, respectively. The reaction rates of these conjugates were roughly parallel with the analgesic activity, decreasing in the order sulfate>glucuronide≒acetate>phosphate>morphine. Furthermore, in the hope that the similar binding of morphine and its 6-conjugates to nucleophilic sites of the brain macromolecule which is closely related to the analgesic receptor might occur, the binding experiment to rat brain homogenates was conducted using morphine and morphine-6-glucuronide which is known to possess stronger analgesic activity than morphine. As the result, the 6-glucuronide showed greater affinity to rat brain but lesser extent of binding to rat liver and bovine serum albumin than morphine.

In a series of metabolic studies on morphine, Yoshimura, *et al.*[2,3] found for the first time that in addition to morphine-3-glucuronide, the major metabolite of morphine, the isomeric 6-glucuronide was also excreted into the urine of several mammalian species including men as one of the minor metabolites of morphine. Unexpectedly, this morphine-6-glucuronide revealed much stronger analgesic activity than the parent compound, morphine, in spite of its highly polar character.[4] Subsequent examination of morphine-3- and 6-sulfate indicated that only the 6-sulfate possessed a strong analgesic activity comparable to that of the 6-glucuronide.[5] Apart from these pharmacological studies, Stork and Clark[6] reported chemical reaction of codeine tosylate with nucleophilic reagents such as piperidine or lithium bromide. By substitution reaction with these nucleophiles, codeine tosylate was converted easily to 6-piperidino-6-deoxycodeine or 8-bromo-6-deoxy-Δ^6-codeine, respectively.

Considering above pharmacological and chemical findings together, we postulated that morphine-6-conjugates which induced potent analgesia might also undergo the similar substitution reaction at C_6 or C_8 with higher reactivity than that of morphine itself. If this occurs in fact, it would not be nonsensical to consider that C_6 or C_8 of morphine molecule combines spontaneously or enzymatically to nucleophilic sites of the certain macromolecules in the brain (the analgesic receptor in another word) and this covalent binding plays some important role in exerting analgesia.

The present investigation was undertaken in order to confirm whether morphine-6-conjugates combine with nucleophilic reagents at C_6 or C_8 more easily than morphine or not

1) Location: *a) 3-1-1 Maidashi, Higashi-ku, Fukuoka; b) 1-14 Bunkyo-machi, Nagasaki; c)* Present address: *Central Research Laboratory, Mitsubishi Chemical Industries, Ltd., 290 Hisamoto, Takatsu-ku, Kawasaki.*

2) H. Yoshimura, K. Oguri, and H. Tsukamoto, *Biochem. Pharmacol.*, **18**, 279 (1969).
3) K. Oguri, S. Îda, H. Yoshimura, and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **18**, 2414 (1970).
4) K. Shimomura, O. Kamata, S. Ueki, S. Îda, K. Oguri, H. Yoshimura, and H. Tsukamoto, *Tohoku J. Exptl. Med.*, **105**, 45 (1971).
5) M. Mori, K. Oguri, H. Yoshimura, K. Shimomura, O. Kamata, and S. Ueki, *Life Sci.*, **11**, part I, 525 (1972).
6) G. Stork and F. H. Clark, *J. Am. Chem. Soc.*, **78**, 4619 (1956).

and then to obtain a clue to the mechanism of analgesic action of morphine or its tolerance development. For this purpose, binding of morphine and the 6-glucuronide to rat brain homogenates was also studied, comparing with binding to rat liver homogenates and bovine serum albumin (BSA) as control macromolecules.

Materials and Methods[7]

Synthesis of 3-Acetyl-6-tosylmorphine (II)——To a solution of 0.1 g (0.305 mmole) of 3-acetylmorphine (I) which was prepared by the method of Welsh,[8] in 0.5 ml of dry pyridine, was added a solution of 0.7 g (3.68 mmoles) of *p*-tosyl chloride in 0.5 ml of dry pyridine. It was stirred for 24 hr at room temperature, poured onto 10 ml of ice-water with rapid stirring and shaken with diethyl ether to remove excess of *p*-tosyl chloride after acidification with 10% HCl. The aqueous layer was made alkaline with conc. NH_4OH and extracted with diethyl ether. The extract was dried over anhyd. Na_2SO_4 and the solvent was evaporated to yield crystalline material. It was recrystallized from MeOH–H_2O to give 70 mg of 3-acetyl-6-tosylmorphine (II), mp 178—179°. *Anal.* Calcd. for $C_{26}H_{27}O_6NS$: C, 64.85; H, 5.65, N, 2.91. Found: C, 64.55; H, 5.72; N, 3.07. UV λ_{max}^{EtOH} nm: 275 (shoulder), 283. IR ν_{max}^{KBr} cm^{-1}: 1755, 1765 (phenol acetate). NMR (in CF_3COOH) δ: 7.88 (2H, doublet, o,o′-H in *p*-tolyl), 7.38 (2H, doublet, m,m′-H in *p*-tolyl), 6.92 (2H, AB-quartet, C_1–H and C_2–H), 6.16 (1H, multiplet, C_7–H), 5.63 (1H, quartet, C_8–H), 5.22 (1H, singlet, C_5–H), 4.52 (1H, doublet, C_6–H), 4.38 (1H, quartet, C_{14}–H). Mass Spectrum *m/e*: 481 (M^+). The hydrochloride of this 3-acetyl-6-tosylmorphine was recrystallized from MeOH to give colorless prisms, mp 214—215°. *Anal.* Calcd. for $C_{26}H_{27}O_6NS \cdot MeOH \cdot HCl$: C, 58.91; H, 5.82; N, 2.54. Found: C, 58.81; H, 5.70; N, 2.54.

Synthesis of 3-Acetyl-6-chloro-6-deoxymorphine (III)——A solution of 0.5 g of 3-acetyl-6-tosylmorphine and 0.18 g of LiCl in 20 ml of acetone was refluxed under anhydrous condition for 4 hr. The cooled solution was poured onto 60 ml of H_2O, and the resulting suspension was extracted with benzene. The extract was dried over anhyd. Na_2SO_4 and the solvent was evaporated to dryness to give crystalline material. It was recrystallized from EtOH to give 0.3 g of colorless prisms of 3-acetyl-6-chloro-6-deoxymorphine, mp 165—167°.[9] *Anal.* Calcd. for $C_{19}H_{20}O_3NCl$: C, 66.01; H, 5.79; N, 4.05. Found: C, 66.02; H, 5.79; N, 3.94. UV λ_{max}^{EtOH} nm: 275 (shoulder), 283. IR ν_{max}^{KBr} cm^{-1}: 1756, 1765 (phenol acetate). NMR (in CF_3COOH) δ: 6.94 (2H, AB-quartet, C_1–H and C_2–H), 6.22 (1H, multiplet, C_7–H), 5.64 (1H, quartet, C_8–H), 5.26 (1H, singlet, C_5–H), 4.54 (1H, doublet, C_6–H), 4.36 (1H, multiplet, C_{14}–H). Mass Spectrum *m/e*: 345 (M^+), 347 ($M^+ + 2$).

Synthesis of 6-Chloro-6-deoxymorphine (IV)——III (0.1 g) was dissolved in a small quantity of H_2O. This solution was stirred for 2—3 hr at room temperature and extracted with $CHCl_3$–isoPrOH (3: 1, v/v) after adjusting the pH to 8.0. Drying over anhyd. Na_2SO_4 and evaporation of the solvent, it gave 6-chloro-6-deoxymorphine (IV) which was recrystallized from MeOH to give 0.08 g of colorless prisms, mp 187—189°.[10] *Anal.* Calcd. for $C_{17}H_{18}O_2NCl$: C, 67.21; H, 5.93; N, 4.61. Found: C, 67.26; H, 6.07; N, 4.21. UV λ_{max}^{EtOH} nm: 286. IR ν_{max}^{KBr} cm^{-1}: 3430 (OH). NMR (in CF_3COOH) δ: 6.87 (2H, AB-quartet, C_1–H and C_2–H), 6.20 (1H, quartet, C_7–H), 5.65 (1H, quartet, C_8–H), 5.24 (1H, singlet, C_5–H), 4.56 (1H, quartet, C_6–H), 4.34 (1H, multiplet, C_{14}–H). Mass Spectrum *m/e*: 303 (M^+), 305 ($M^+ + 2$).

Synthesis of 8-Piperidino-6-deoxy-Δ^6-morphine (V)——A solution of 0.27 g of II and 1.0 ml of piperidine in 10 ml of benzene was refluxed for 3 hr on a boiling water bath. It was then extracted 3 times with 1 ml each of dil. HCl solution. The aqueous layer was washed once by shaking with diethyl ether and made alkaline (pH 10) with dil. NaOH solution. It was extracted with diethyl ether, and the extract was washed repeatedly with a small quantity of H_2O. This was dried over anhyd. K_2CO_3, and the solvent was evaporated to dryness to yield 0.21 g of colorless prisms (V), mp 220—224°, after recrystallization from diethyl ether, showing existence of phenolic hydroxyl group by coloration with $FeCl_3$ reagent. This compound was similarly obtained by the reaction of III or IV with piperidine in a good yield. *Anal.* Calcd. for $C_{22}H_{28}O_2N_2 \cdot 1/2(C_2H_5)_2O$: C, 74.00; H, 8.54; N, 7.19. Found: C, 73.67; H, 8.87; N, 7.04. UV λ_{max}^{EtOH} nm: 286. IR ν_{max}^{KBr} cm^{-1}:

7) All melting points were measured on Yanagimoto micro-melting point apparatus and uncorrected. Infrared (IR) and ultraviolet (UV) spectra were obtained with a JASCO DS-701G and a Shimadzu SV-50-A spectrophotometers, respectively. Nuclear magnetic resonance (NMR) spectra were taken at 100 MHz on a JEOL PS-100 spectrometer and chemical shifts were given in δ (ppm) scale with tetramethylsilane as internal standard. Mass spectra were recorded on JMS-01SG mass spectrometer with an accelerating potential of 6.2 kV, an ionizing potential of 75 eV and a source temperature of 100—150°.

8) L.H. Welsh, *J. Org. Chem.*, **19**, 1409 (1954).

9) In connection with this compound, L. Ach and H. Steinbock reported acetyl-β-chloromorphide, mp 163°, in *Chem. Ber.*, **40**, 4281 (1907). This was obtained by acetylation of β-chloromorphide, mp 188°, which was prepared by chlorination of morphine with HCl at 65° in a sealed tube.

10) In connection with this compound, R. Pschorr reported α-chloromorphide, mp 192°, which was obtained by the chlorination of morphine with HCl at room temperature in a sealed tube, in *Chem. Ber.*, **39**, 3130 (1906), and L. Ach and H. Steinbock[9] also reported the synthesis of β- chloromorphide, mp 188°.

3440 (OH). NMR (in CF_3COOH) δ: 6.96 (2H, AB-quartet, C_1–H and C_2–H), 6.28 (1H, quartet, C_6–H), 6.00 (1H, quartet, C_7–H), 5.30 (1H, doublet, C_5–H), 4.84 (1H, multiplet, C_8–H). Mass Spectrum *m/e*: 352 (M^+).

Synthesis of Morphine-6-conjugates——Morphine-6-glucuronide,[11] 6-sulfate,[5] 6-phosphate[12] and 6-acetate[13] were prepared by the previously reported methods, respectively.

Reaction of Morphine-6-conjugates with Lithium Chloride or Piperidine——A Solution of 0.2 mmole of morphine-6-conjugates (IIa,b,c,d) and 1.0 mmole of LiCl in 5 ml of acetone or 8 mmoles of piperidine in 5 ml of benzene was refluxed under anhydrous condition for 4 or 3 hr, respectively, on a water bath. The cooled reaction mixture was poured onto 15 ml of H_2O and resulting suspension was extracted 3 times with benzene. The extract was dried over anhyd. Na_2SO_4 and the solvent was evaporated to give 6-chloro-6-deoxymorphine (IV) or 8-piperidino-6-deoxy-Δ^6-morphine (V).

Synthesis of ^{14}C-Labeled Morphine and Morphine-6-glucuronide——^{14}C-Morphine·HCl (specific activity: 1.03 μCi/mg) was prepared from normorphine and ^{14}C-paraformaldehyde according to the method of Anderson and Wood.[14] ^{14}C-Morphine-6-glucuronide (specific activity: 0.7 μCi/mg) was synthesized from ^{14}C-morphine described above according to the previously reported method.[15] These two radioactive samples were dissolved in 0.9% saline in a concentration of 3.5×10^{-5} mole/ml and used for binding experiment.

Tissue Preparation and Binding Experiment——Male rats of the Donryu strain weighing 150—180 g were stunned, exsanguinated, and the brains and the livers were immediately excised. These tissues were washed with cold 1/15 M phosphate buffer (pH 7.4) to remove blood and then homogenized in 4 volumes of the same buffer with a Potter-Elvehjem type homogenizer. To 1.0 ml of each homogenate was added certain amounts of a solution of ^{14}C-morphine or ^{14}C-morphine-6-glucuronide in 0.9% saline described above, and the final volume was adjusted to 1.2 ml with 1/15 M phosphate buffer (pH 7.4). The mixture was incubated with gentle agitation at 37° for 15 min in a metabolic shaker.

An aliquot of 1.0 ml of the reaction mixture and 1.0 ml of 1/15 M phosphate buffer (pH 7.4) were transferred to cellulose tube (Visking, size 18/32) and dialyzed against 100 ml of 1/15 M phosphate buffer at 0—5° for 6 days on a magnetic stirrer, the buffer solution being renewed every other day. Radioactivity of the inner dialysis solution was then measured to calculated the amount of morphine or morphine-6-glucuronide bound to the tissue homogenates as described below. Similar binding experiment was carried out by use of bovine serum albumin (BSA, Armour, fraction V). In this case a solution of 50 mg per ml 1/15 M phosphate buffer was used instead of the homogenates described above.

Radioactivity Measurement——An aliquot (0.5 ml) of the inner dialysis solution described above was digested with 2 ml of Bio-solv (Beckman, BBS-3) for 20 hr in the dark, and the radioactivity in this solution was determined, after addition of 2 drops of 30% ascorbic acid and 15 ml of toluene phosphor containing 2,5-diphenyloxazole (0.4%) and 1,4-bis[2-(4-methyl-5-phenyloxazoyl)]benzene (0.02%) in toluene, with an Aloka liquid scintillation spectrometer (model 502, Japan Radiation of Medical Electronics, Inc., Tokyo), being corrected for quenching by an external standard method using Ra^{226}.

Results and Discussion

Reaction of Morphine-6-conjugates with Lithium Chloride and Piperidine

Before examining reactivity of morphine-6-conjugates with nucleophilic reagents such as lithium chloride and piperidine, the proposed products, chlorine- and piperidine-substituted morphine derivatives, were firstly attempted to synthesize according to the reaction routes as described in Chart 1.

The starting material, 3-acetylmorphine (I), which was easily obtained by the method of Welsh,[8] was allowed to react with an excess of *p*-tosyl chloride in dry pyridine to afford 3-acetyl-6-tosylmorphine (II). In this reaction, presence of a trace of water or use of more diluted solution of *p*-tosyl chloride resulted in the formation of 3-acetyl-6-chloro-6-deoxymorphine (III). This reaction seemed to proceed through substitution reaction of once formed II with chloride ion which should also be present in the reaction mixture. The standard sample of this compound (III) was obtained by the substitution reaction of II with lithium chloride in a good yield. Definite evidence that chlorine atom is located at C_6 of the compound (III)

11) H. Yoshimura, K. Oguri, and H. Tsukamoto, *Chem. Pharm. Bull.* (Tokyo), **16**, 2114 (1968); *idem*, *ibid.*, **18**, 209 (1970).
12) M. Mori, K. Oguri, H. Yoshimura, and O. Kamata, *Yakugaku Zasshi*, **93**, 1302 (1973).
13) C. I. Wright, *J. Pharmacol. Exptl Therap.*, **71**, 164 (1941).
14) K.S. Anderson and A.L. Wood, *J. Org. Chem.*, **24**, 274 (1957).
15) H. Yoshimura, S. Îda, K. Oguri, and H. Tsukamoto, *Biochem. Pharmacol.*, **22**, 1423 (1973).

was provided by the NMR spectrum which was similar to those of 3-acetyl-6-tosylmorphine (see Materials and Methods) and morphine [in CF_3COOH, δ: 6.90 (2H, AB-quartet, C_1–H and C_2–H), 6.00 (1H, multiplet, C_7–H), 5.46 (1H, quartet, C_8–H), 5.24 (1H, doublet, C_5–H), 4.66 (1H, quartet, C_6–H), 4.36 (1H, multiplet, C_{14}–H)]. This structure was also supported by the decoupling experiments of III. Thus, irradiation at δ 6.22 (C_7–H) resulted in the alteration of the quartet at δ 5.64 (C_8–H) and the doublet at δ 4.54 (C_6–H) to a doublet and a singlet, respectively, while irradiation at δ 5.64 (C_8–H) varied the multiplet at δ 6.22 (C_7–H) to a doublet. The compound (III) was very labile and easily hydrolyzed in H_2O, dil. NH_4OH or CF_3COOH at room temperature to 6-chloro-6-deoxymorphine (IV).

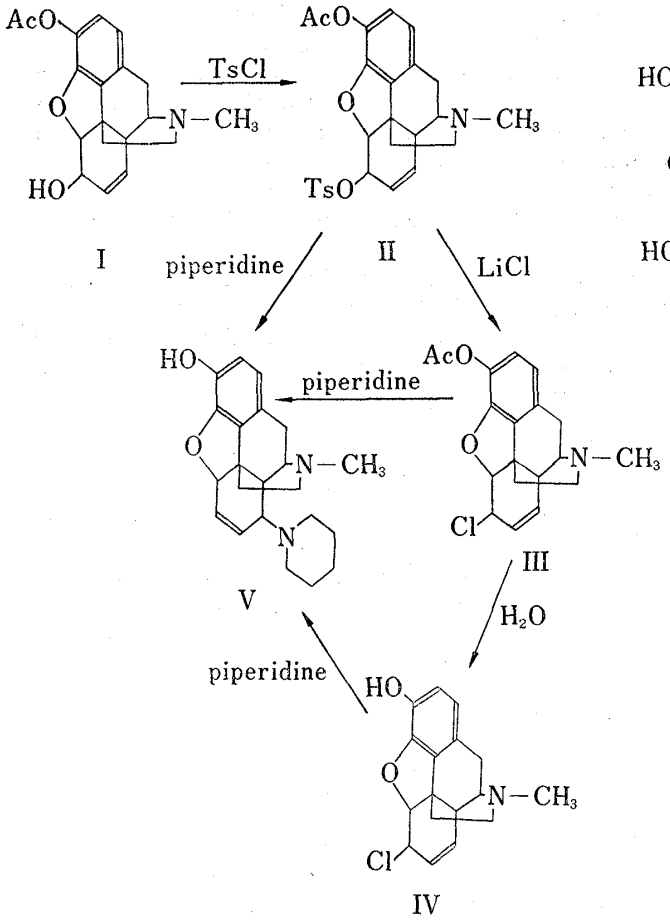
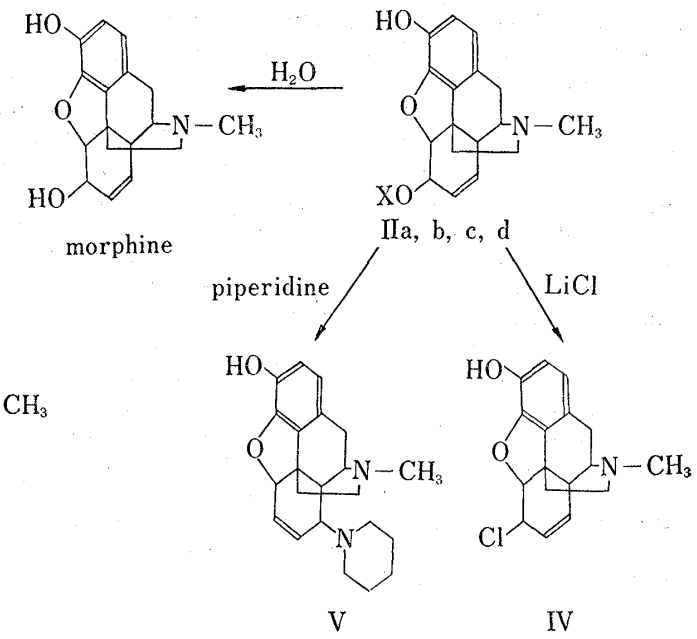


Chart 1



IIa : sulfate (X=–SO_3H)
IIb : glucuronide (X=glucuronic acid residue)
IIc : acetate (X=–$COCH_3$)
IId : phosphate (X=–PO_3H_2)

Chart 2

Table I. Reaction of Morphine-6-conjugates with Lithium Chloride and Piperidine

Reagent	Morphine-6-conjugates	Unchanged recovery (%)	Products (IV, V) yield (%)	Morphine yield (%)
LiCl	sulfate	0	50—55	0
	glucuronide	0	40—45	10—15
	morphine	97—98	0	—
Piperidine	sulfate	10—15	45—50	0
	glucuronide	10—15	30—35	5—10
	acetate	0	30—35	45—50
	phosphate	60—65	10—15	0
	morphine	95—98	0	—

See Materials and Methods for reaction condition.

On the other hand, reaction of II, III or IV with piperidine provided 8-piperidino-6-deoxy-Δ^6-morphine (V), but not 6-piperidino-6-deoxymorphine. The structure of this compound was also ascertained by the NMR spectrum (see Materials and Methods) and also decoupling experiments as follows; irradiation at δ 6.28 (C_6–H) resulted in the alteration of the doublet at δ 5.30 (C_5–H) to a singlet, while irradiation of the latter signal varied the former quartet (C_6–H) to a doublet. With reference to these compounds, Stork and Clark[6] described the substitution reaction of codeine tosylate with piperidine in boiling benzene or with lithium chloride in boiling acetone. In these cases, however, they obtained 6-piperidino-6-deoxycodeine or 6-chloro-6-deoxycodeine which underwent thermal rearrangement to give 8-chloro-6-deoxy-Δ^6-codeine, respectively.

Next, attempt was made to explore the reactivity of morphine-6-conjugates with lithium chloride or piperidine in boiling acetone or benzene, respectively (see Chart 2). The results are summarized in Table I.

As can be seen in Chart 2 and Table I, all the 6-conjugates examined were convertible to 6-chloro-6-deoxymorphine or 8-piperidino-6-deoxy-Δ^6-morphine by the substitution reaction. Although the reaction rates decreased in the order, sulfate>glucuronide≒acetate>phosphate, C_6 position of these conjugates revealed greater reactivity to nucleophilic reagents than morphine itself. Morphine did not give any products under the reaction condition used, and mostly recovered as unchanged material. However, heating with conc. HCl in a sealed tube, morphine was easily converted to 6-chloro-6-deoxymorphine in a good yield. It is very interesting that among above 6-conjugates the 6-sulfate and glucuronide which induce stronger analgesia have shown higher substitution reactivity. The result with the 6-acetate was also very suggestive, because two groups of workers[16,17] reported independently that the 6-acetate and heroin were virtually equipotent in pharmacological activity and therefore heroin might act principally as this acetate. Furthermore, the 6-phosphate exerted only a little substitution reactivity in parallel with its analgesic activity (the same level as morphine).[12] These results indicate that the introduction of an electronattracting group at the C_6 position of morphine molecule increases the reactivity of either C_6 or C_8 to nucleophilic reagents and strongly suggest that the similar binding of morphine to nucleophilic sites of the analgesic receptor or of the brain macromolecule, which is closely related to it, may occur. Although no evidence is available at the present, such reaction is postulated to proceed not only chemically, but also enzymatically in the body.

Binding of Morphine and Morphine-6-glucuronide to Rat Brain

In order to confirm the plausibility of above presumption, covalent binding of morphine and the 6-glucuronide to the brain macromolecules was examined. For this purpose, the

Table II. Binding of Morphine and the 6-Glucuronide (M-6-G) to Rat Brain and Liver, and Bovine Serum Albumin (BSA)

Substrate added ($\times 10^{-6}$ moles)	Amount of drug ($\times 10^{-8}$ moles) bound to					
	Brain (200 mg)		Liver (200 mg)		BSA (50 mg)	
	M-6-G	Morphine	M-6-G	Morphine	M-6-G	Morphine
0.35	0.77	0.32	—	—	—	—
0.70	1.33	0.59	0.34	0.63	0.53	0.94
1.75	2.46	1.45	1.39	1.97	1.47	1.72
3.50	4.92	2.96	—	—	—	—

Values are expressed as mean of 3 experiments. See Materials and Methods for reaction condition.

16) N.B. Eddy and H.A. Howes, *J. Pharmacol. Exptl. Therap.*, **53**, 430 (1935).
17) C.I. Wright and F.A. Barbour, *J. Pharmacol. Exptl. Therap.*, **54**, 25 (1935).

^{14}C-labeled drugs were incubated with brain homogenates of rats at 37° for 15 min, and the incubation mixture was dialyzed exhaustively against 1/15 M phosphate buffer for 6 days. From the radioactivity in the inner dialysis solution, the amount of drugs bound to the brain macromolecules was calculated. At the same time, the binding to liver homogenates of rats and to BSA was also studied in order to learn the extent selectivity of this binding.

As the result, significant amounts of morphine and the 6-glucuronide showed to bind to the brain macromolecules and these bindings were not saturated in the reaction conditions used (Table II).

Since the radioactivity bound to the macromolecules can no longer be released by further dialysis, this binding is perhaps covalent, although it is not certain whether it occurs between the nucleophilic sites of the macromolecules and C_6 or C_8 of morphine molecule or not. Table II also indicated that the 6-glucuronide revealed greater extent of binding to the brain macromolecules than morphine. Perhaps most of these bindings might be non-specific and only a very little part might concern with the specific binding to the analgesic receptor. In accordance with this presumption, bindings of morphine to rat liver and BSA occurred to greater extent than the respective binding of the 6-glucuronide. It was also found that the 6-glucuronide showed greater affinity to rat brain than rat liver, while the result with morphine was the reverse. Although these *in vitro* findings are not directly applicable to explain the *in vivo* phenomenon, it is very interesting that the 6-glucuronide which has greater analgesic activity shows more specific binding to the brain macromolecules than morphine.

Acknowledgement A part of this work was financially supported by the Naito Research Grant for 1974, to which the authors thanks are due. They also wish to thank to the members of Analytical Room of this Faculty for microanalyses and spectral measurements.