Subcellular Distribution of N-methyl-c^{14} morphine in Brain and Liver of Normal Mice and Naloxone Treated Mice.

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Since differential centrifugation and sucrose gradient ultracentrifugation techniques bave been used to separate subcellular organelles and components of brain tissue, experiments have been designed to investigate the initial action site of morphine by studying its subcellular distribution. ①②③ As much morphine research has been done by using mice, an understanding of the intracellular distribution of this drug on this species is highly desirable. In this communication, the subcellular distribution of N-methyl-c^{14} morphine in the brain and liver of normal mice and naloxone treated mice are reported.

Materials and Methods.

N-methyl-c^{14} morphine solution: N-methyl-c^{14} morphine HCl (0.5mCi per ampoule, specific activity 152 μCi/mg) was purchased from Amersham/Searle Corporation. Its purity and radioactivity was checked by descending paper chromatography (benzene: methanol: isoamyl alcohol : water: pyridine = 9 : 7 : 3 : 2 : 1) before use. One ampoule of this N-methyl-c^{14} morphine HCl was dissolved in 10 ml distilled water which contained 10 mg non-radioactive morphine sulphate. The radioactivity of the final solution was 50 μCi/ml.

Experimental animals: Swiss webster mice, weighing about 20 g, of both sexes, were used. 5 normal mice, each received 0.2 ml N-methyl-c^{14} morphine solution (10 μCi per mouse, 10 mg morphine base per kg body weight) via tail vein injection, were killed 15 minutes after injection by decapitaion Brain and liver were removed immediately. Blood was also collected for Sephadex column chromatography study. 5 naloxone treated mice, which received 10 mg/kg body weight of naloxone HCl (N-allylnoroxymorphone HCl) by intraperitoneal injection 15 minutes before i.v. injection of N-methyl-c^{14} morphine solution, were killed 15 minutes after morphine injection.

Subcellular fractionation of brain tissues: Brains were dissected into three parts, viz., cerebral cortex, brain stem with hypothalamus, and cerebellum. Each part of 5 mice brains was pooled together and homogenized in 0.32 M sucrose solution in a ratio of 1:10 w/v with a glass homogenizer and Teflon pestle for 2 minutes. The crude nuclear fraction was sedimented at 900 × g for 10 minutes in the ss-34 angle head of the Sorvall centrifuge. The supernatant was decanted and the pellet resuspended in 5 ml of 0.32 M sucrose solution and centrifuged a second time; this procedure was performed twice. The supernatant and washings were combined and centrifuged at 11,500 × g for 20 minutes to sediment the crude mitochondrial fraction which was subsequently washed in a manner similar to that for the nuclear fraction. The supernatant and washings were centrifuged at 100,000 × g for 30 minutes in the No. 40 angle head of Spinco model L ultracentrifuge. The pellet obtained in this manner was microsomal fraction, and the supernatant was the soluble fraction. Subfractionation of crude mitochondrial fraction was carried out according to the discontinuous sucrose gradient ultracentrifugation method described by DeRobertis. ④ Crude mitochondrial pellet was suspended in 3 ml 0.32 M sucrose solution. The suspension was carefully layered on the top of a discontinuous sucrose gradient, which was made of four 7-ml layers of 0.8 M, 1.0 M, 1.2 M, and 1.4 M sucrose solutions in a 35-ml capacity cellulose nitrate centrifuge tube, and centrifuged at 20 Krpm for 2 hours in a SW 25.1 rotor of Spinco model L ultracentrifuge. 5 components of crude mitochondrial fraction separated

by this method, A, B, C, D, and E from the top of centrifuge tube, were designated by DeRobertis as myelin, heterogeneous, cholinergic synaptosome, non-cholinergic synaptosome, and mitochondria, respectively. The content of the cellulose nitrate centrifuge tube was then separated and collected from the top of the tube into 30 1-ml fractions by pushing up with a 55% sucrose chasing solution from the bottom of the tube in an ISCO fractionator. Component E (mitochondria) which packed tightly to the bottom of the centrifuge tube could not be collected by this process. Separation of synaptic vesicles and other components of synaptosome was performed by the Whittaker method ⑤ Component C (cholinergic synaptosomes) obtained from the crude mitochondrial fraction of 5 mice whole brains was diluted with 1 volume of distilled water and centrifuged at 15 Krpm for 20 minutes in the ss-34 angle head of Sorvall centrifuge. The synaptosome pellet obtained was lysed by suspension in 5 ml distilled water overnight. The lysate was carefully layered on the top of a discontinuous sucrose gradient, which was made of five 5-ml layers of 0.4 M, 0.6 M, 0.8 M, 1.0 M, and 1.2 M sucrose solutions in a 35 ml capacity cellulose nitrate centrifuge tube, and centrifuged at 25 Krpm for 2 hours in a SW 25.1 rotor of Spinco model L ultracentrifuge. Seven components separated from the lysate were designated by Whittaker as O (soluble cytoplasm), D (synaptic vesicles), E (membrane), F (membrane), G (membrane), H (incompletely disrupted synaptosomes), and I (intra-terminal mitochondria) from the top of the centrifuge tube. The content of this centrifuge tube was also separated and collected from the top into 30 1-ml fractions in an ISCO fractionator. Component I (intra-terminal mitochondria) could not be collected by this process since it sedimented to the bottom of the centrifuge tube. All processes described above were performed at 4° C.

Subcellular fractionation of liver tissue: Liver was homogenized in 0.88 M sucrose solution at 4° C in a ratio of 1:10 w/v with a glass homogenizer and Teflon pestle for 2 minutes. The homogenate was fractionated at 4° C into nuclear, mitochondrial, microsomal, and soluble fractions by differential centrifugation according to the method of Hogeboom et al. ⑥

Counting procedure for radioactivity: 0.2 ml 10 N NaOH was added to the subcellular component sample in a counting vial and diluted with distilled water to make it 2 N NaOH and final volume 1 ml, heated in a 80° C water bath for 30 minutes, cooled to room temperature, and neutralized by adding conc. HCl dropwise. Suspended in 2 ml BBS-2 (Beckman Biosolv. No. 184-982) and let stand for 5 to 10 minutes, then added 2 drops of freshly prepared 4% $SnCl_2$ in 0.1 N HCl. 10 ml of toluene-butyl PBD-PBBO scintillation solution (Scintillation Flour, Beckman formula TLA No. 161233) was added into the vial, mixed, and let stand overnight, then counted in Beckman liquid scintillation counter for 10 minutes. Radioactivity of fractions collected from Sephadex column chromatography was determined by adding 0.1 ml of each fraction onto a 1.5×1.5 cm Whatman No. 3 filter paper in a counting vial. After air dried, 5 ml scintillation solution (0.5% PBD and 0.01% POPOP in scintillation grade toluene) was added, and mixed gently, then counted in Beckman liquid scintillation counter for 10 minutes.

Determination of protein: Protein content of subcellular fractions of brain and liver, and fractions obtained from Sephadex column chromatography were determined by the method of Lowry et al. ⑦

Sephadex G-25 column chromatography: A 9×300 mm Sephadex G-25 column was made by packing fine Sephadex G-25 (Pharmacia Fine Chemicals), which had been equilibrated with Tris-HCl buffer solution (PH = 7, ionic strength = 0.05), into a 9 × 400 mm glass column. Sample solution, less than 1 ml, was applied onto the Sephadex column and eluted with the Tris-HCl buffer solution. Each 0.75 ml effluent was collected as a fraction. The

position of morphine-3-monoglucuronide and morphine-3-ethereal sulphate (these two compounds were obtained from Dr. Fujimoto) in the effluent of Sephadex column chromatography was checked by measuring the optical density of each fraction at wavelength 280 mμ in a Gilford spectrophotometer.

Results

Subcellular distribution patterns of radioactivity in the brain tissues: Fig. 1 indicates the percentage distribution of radioactivity in the subcellular fractions of normal mice brain tissues, 15 minutes after i.v. injection of N-methyl-c^{14} morphine solution (10 μCi per mouse, 10 mg morphine base per kg body weight). Most of the radioactivity (65 to 70%) was found in the soluble fraction, and only 30% radioactivity was associated with the particulate fractions. The crude nuclear fraction had the least amount of radioactivity (less than 5%). In cerebral cortex, the microsomal fraction had the highest value of specific radioactivity (DPM/mg protein) among the three particulate fractions; while in the cerebellum, the specific radioactivity of crude mitochondrial fraction was higher than that of microsomal fraction. In naloxone treated mice (10 mg naloxone base per Kg body weight i. p. injection 15 minutes before i.v. injection of N-methyl-c^{14} morphine solution), the entry of morphine into brain tissues was not interfered, since the specific radioactivity values of brain tissue homogenates of normal mice and naloxone treated mice were almost identical. (Tab. 1) The subcellular radioactivity distribution in brain tissues of naloxone treated mice is shown in Fig. 2. About 65% radioactivity of each brain tissue was also found in the soluble fraction. In the presence of naloxone, the specific radioactivity of the crude mitochondrial fraction of brain tissues was not decreased at all. Only in brain stem with hypothalamus and in cerebellum, the specific radioactivity of microsomal fraction was reduced.

Subcellular distribution pattern of radioactivity in liver: The percentage distribution pattern of radioactivity and value of specific radioactivity in the subcellular fractions of mice liver are shown in Fig. 3. The specific radioactivity in liver tissue was much higer than that in brain tissues, but the distribution patterns of radioactivity of liver and of brain tissues were similar. About 70% of radioactivity of liver tissue was also found in the soluble fraction, and the remaining 30% radioactivity was more or less evenly distributed among the three particulate fractions. Both of the subcellular distribution patterns of radioactivity in normal mice liver and in naloxone treated mice liver were similar, except the specific radioactivity of the latter was lower (70% of normal mice).

Distribution of radioactivity in the components of crude mitochondrial fraction of brain tissues: Fig. 4 shows the distribution of radioactivity in the components of crude mitochondrial fraction of normal mice brain tissues. Among the four components (the pure mitochondria were sedimented to the bottom of the centifuge tube and could not be pushed out by the sucrose chasing solution), component A (myelin) and component C (cholinergic synaptosomes) had much higher radioactivity than that of component B (heterogeneous) and that of component D (non-cholinergic synaptosomes). Amounts of radioactivity in component A and in component C of cerebral cortex were equal (30% of crude mitochondrial fraction). In the brain stem and hypothalamus, 40% radioactivity of crude mitochondrial fraction was in component A and 20% radioactivity of crude mitochondrial fraction was in component C. Since the radioactivity was proportional to the protein content, the specific radioactivity of component A and of component C was not higher than that of component B

and of component D. The results also showed that the non-particulate soluble portion (fraction No. 1) had high content of radioactivity, the radioactivity was possibly due to free morphine molecules which were originally loosely bound with particulate components or released from ruptured synaptosomes during the period of manipulation, especially during resuspension of the crude mitochondrial pellet in 0.32 M sucrose solution. Distribution of radioactivity in the components of crude mitochondrial fraction of naloxone treated mice brain tissues is presented in Fig. 5. Naloxone did not alter the distribution pattern of radioactivity nor did it reduce the specific radioactivity of any component.

Distribution of radioactivity in the synaptic vesicles and other components of synaptosome: Component C (cholinergic synaptosomes) obtained from whole brains of five N-methyl-c^{14}morphine injected mice was, after osmotic lysis, separated further by discontinuous sucrose gradient ultracentrifugation into seven components. Most of the radioactivity (55 to 60%) of the synaptosomes was found in component O (soluble cytoplasm) and component D (synaptic vesicles). The result of radioactivity distribution among these components is shown in Fig 6.

Sephadex G-25 column chromatography studies: Sephadex G-25 column chromatograms of 1 ml brain soluble fraction, 1 ml liver soluble fraction, and 0.25 ml serum obtained from N-methyl-c^{14}morphine injected mice are shown in Fig. 7. The positions of morphine, morphine-3-monoglucuronide, and morphine-3-ethereal sulphate which would appear in the effluent of the sephadex G-25 column chromatography are shown in Fig. 8. In brain soluble fraction, 92% radioactivity appeared in the effluent from fraction Nos. 31 to 36, the molecular nature of this portion was confirmed to be unchanged morphine by paper chromatography; 8% of radioactivity appeared in the effluent from fraction Nos. 24 to 28, this was most probably morphine glucronide since it could be hydrolyzed and release free morphine by the action of bovine liver beta-glucuronidase (Sigma Type B-1); there was no radioactivity associated with protein. In the liver soluble fraction, most of the morphine was found converted into its conjugated metabolites, and about 3% radioactivity was protein bound. In serum, 21% radioactivity was free morphine, 75% radioactivity was morphine glucuronide, and 4% radioactivity was bound with serum protein.

Discussion

This in vivo subcellular distribution study indicates that 65 to 70% of morphine in mouse brain or liver is located in the soluble fraction of homogenate. The result is consistent with the previous reports. ①②③ Morphine molecule in brain soluble fraction does not bind with protein. Although about 3% radioactivity of liver soluble fraction and 4% radioativity of serum are protein bound, this does not necessarily mean that morphine binds with protein. Direct incubation of N-methyl-c^{14} morphine solution with serum or plasma followed by running it through sephadex G-25 column, no radioactivity was associated with protein. Since morphine can be N-demethylated in liver, the radioactive one-carbon moiety formed from demethylation of N-methyl-c^{14}morphine is most likely the source of protein bound radioactivity.

In components separated from crude mitochondrial fraction of brain tissues, mylin (A) and cholinergic synaptosome (C) fractions have a significant amount of radioactivity In cerebral cortex, radioactivity of mylin and cholinergic synaptosomes are equal, but in brain stem and hypothalamus or cerebellum, radioactivity of myelin is equal to or greater than twice that of cholinergic synaptosomes. The distribution patterns of different regions of brain show that there is a direct relationship between the radioactivity ratio and the

protein content ratio of these two components, and the amount of morphine associated with synaptosomal fraction is dependent on the number of synaptosome presented.

The radioactivity in cholinergic synaptosomal fraction is only 3% of the radioactivity in brain tissue homogenate. Nerve endings of central nervous system has been suspected to be the action site of morphine, but this in vivo distribution study could not demonstrate significant accumulation of morphine in synaptosomal fraction. In the report of Mule' et al., only 1% H^3-morphine in guinea pig brain was found in cholinergic synaptosomal fraction. ③ Dissociation of morphine from particulate cellular components due to its weak binding, or release of morphine from ruptured synaptosomes during homogenizing of brain tissue and resuspension of the crude mitochondrial pellet is a possible reason which may account for the low content of morphine in synaptosomal fraction. In vitro morphine uptake study of Navon et al. ⑧ showed that significant amount of morphine was bound to synaptosome after incubation. Recently, Scrafani et al. ⑨ studied the intracellular H^3-dihydromrphine distribution in rat brain by separation of brain homogenate into subcellular fractions with only one centrifugation through a linear sucrose density gradient. Their data indicated that 15 to 25% of H^3-dihydromorphine was bound to the synaptosomal fraction of different regions of rat brain.

The action mechanism of morphine antagonist had been thought to compete with morphine for cell-receptor sites. Seeking of evidence for this hypothesis was done by studying subcellular morphine distribution in the presence of its antagonist. Mule et al. ③ could not demonstrate any effect of nalorphine on the subcellular H^3-morphine distribution in guinea pig brain. In vitro study of Navon et al. ⑧ shows that uptake of morphine by nuclei and by mitochondria of rat brain was inhibited by nalorphine. Naloxone (N-allylnoroxymorphone) is a potent narcotic antagonist. ⑩ ⑪ In this experiment, naloxone did not affect the enry of morphine into the central nervous system and, the subcellular distribution pattern of morphine.

Summary

15 minutes after i.v. injection of N-methyl-c^{14}morphine (10 μCi per mouse; 10 mg morphine base per Kg body weight), distribution of radioactivity in mice brain tissues and liver has been studied. 65 to 70% radioactivity of brain tissues and liver was found in the soluble fraction. The radioactivity in the crude mitochondrial fraction of different regions of brain ranged from 10 to 20%. In the components of the crude mitochondrial fraction, only myelin (A) fraction and cholinergic synaptosome (C) fraction had a significant amount of radioactivity. The radioactivity in the cholinergic synaptosomal fraction was only 3% of the radioactivity of brain tissue homogenate. Most of the radioactivity (55 to 60%) of the cholinergic synaptosomes was found in soluble cytoplasm (○) and synaptic vesicles (D). Sephadex G-25 column chromatography studies indicated that 92% radioactivity in brain soluble fraction was unchanged morphine, 8% radioactivity was morphine glucuronide, and no radioactivity was associated with protein. I.p. injection of naloxone (10 mg naloxone base per Kg body weight) 15 minutes prior to N-methyl-c^{14}morphine injection had no effect on the entry of morphine into the central nervous system and the subcellular distribution pattern of morphine.

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Tab. 1. Specific radioactivity of normal mice brain tissue homogenates and naloxone treated mice brain tissue homogenates.

Brain tissue	Specific radioactivity (DPM/mg protein)	
	Normal	Naloxone treated
Cerebral cortex	237	253
Brain stem and hypothalamus	196	193
Cerebellum	241	248

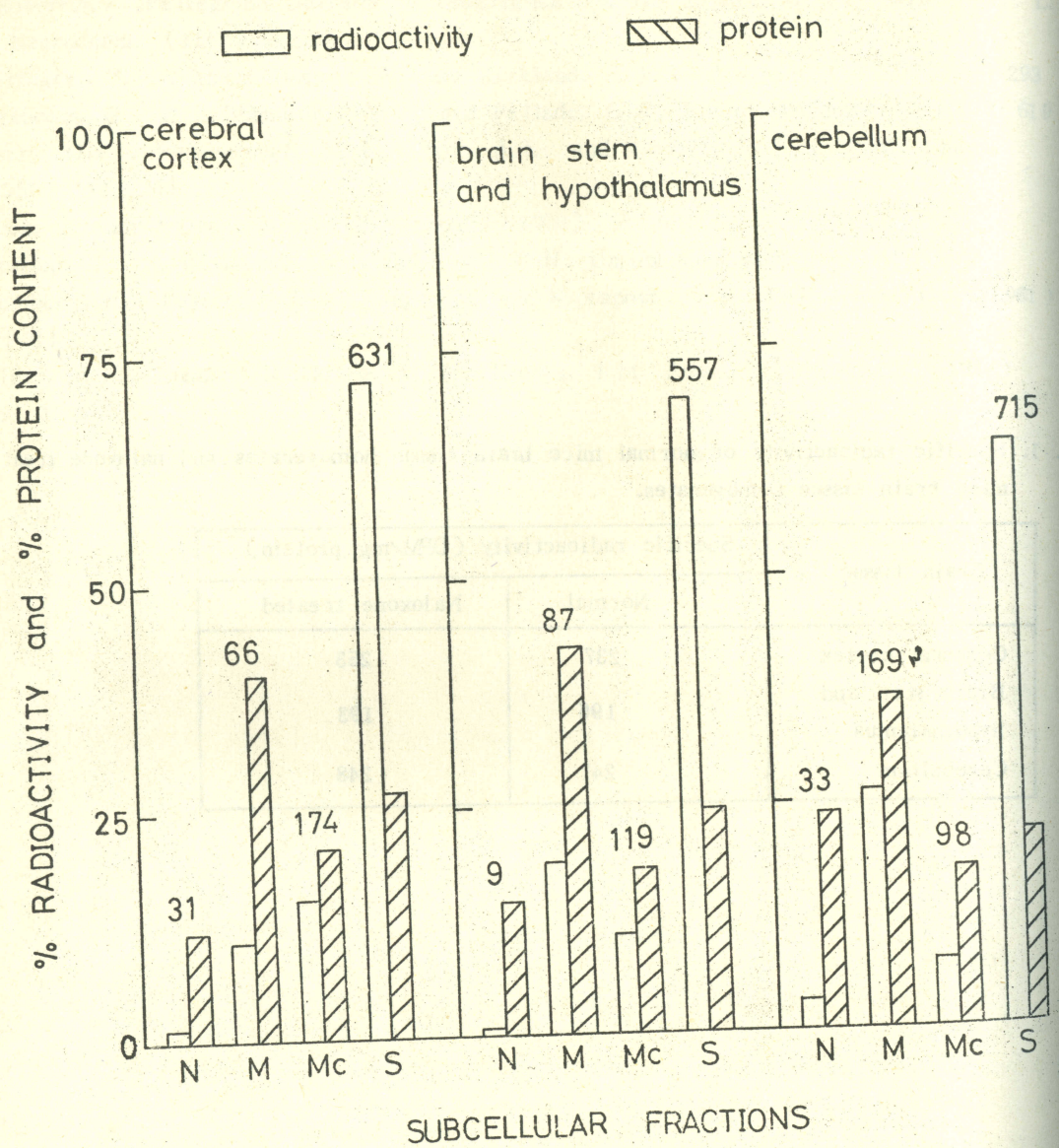

Fig. 1. Subcellular distribution patterns of radioactivity in the brain tissues of normal mice. N: crude nuclear fraction; M: crude mitochondrial fraction; Mc: microsomal fraction; S: soluble fraction. The numbers on the top of bars are the specific radioactivity (DPM / mg protein) of each fraction.

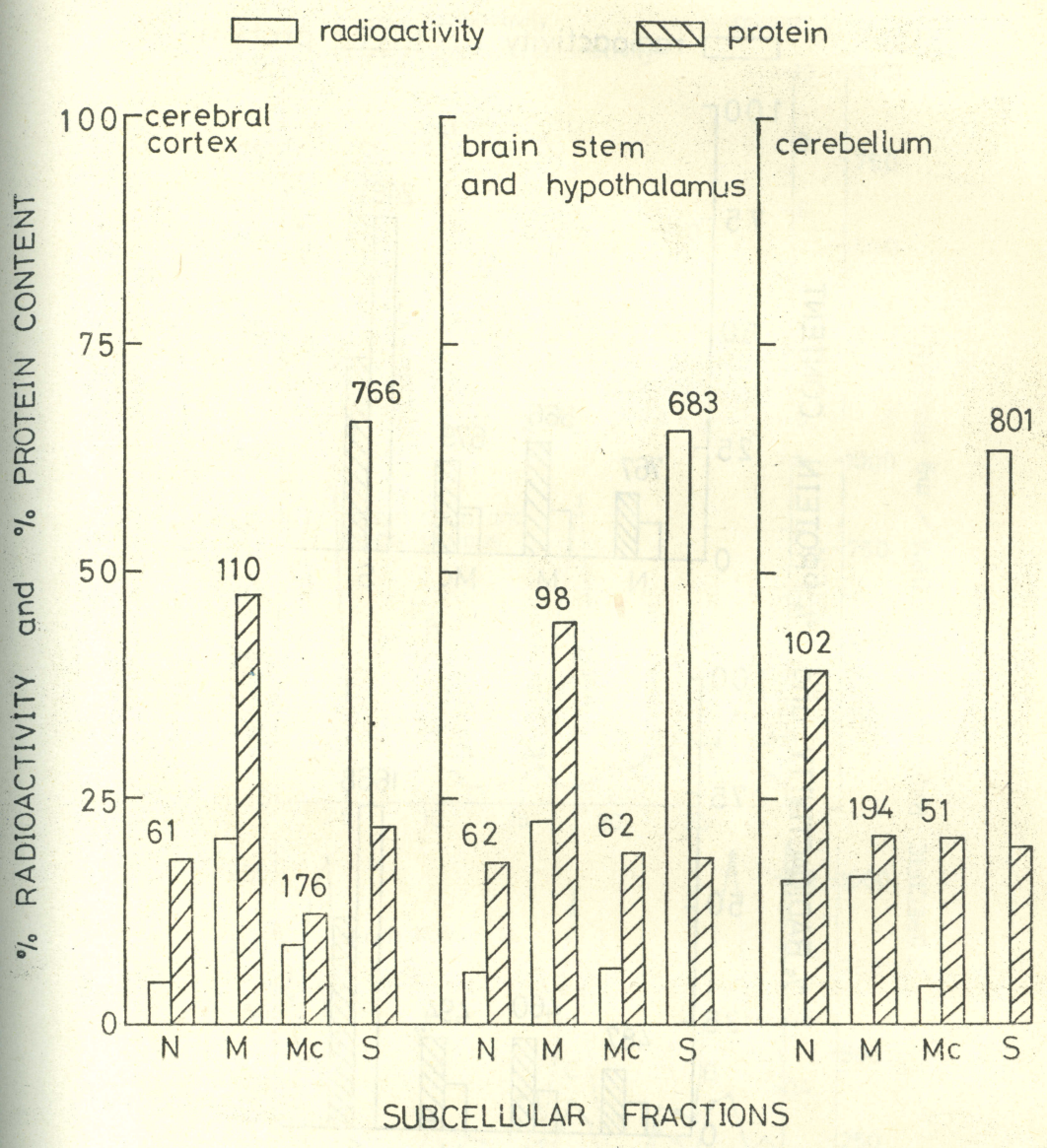

Fig. 2. Subcellular distribution patterns of radioactivity in the brain tissues of naloxone treated mice. N: crude nuclear fraction; M: crude mitochondrial fraction; Mc: Microsomal fraction; S: soluble fraction. The numbers on the top of bars are the specific radioactivity (DPM/mg protein) of each fraction.

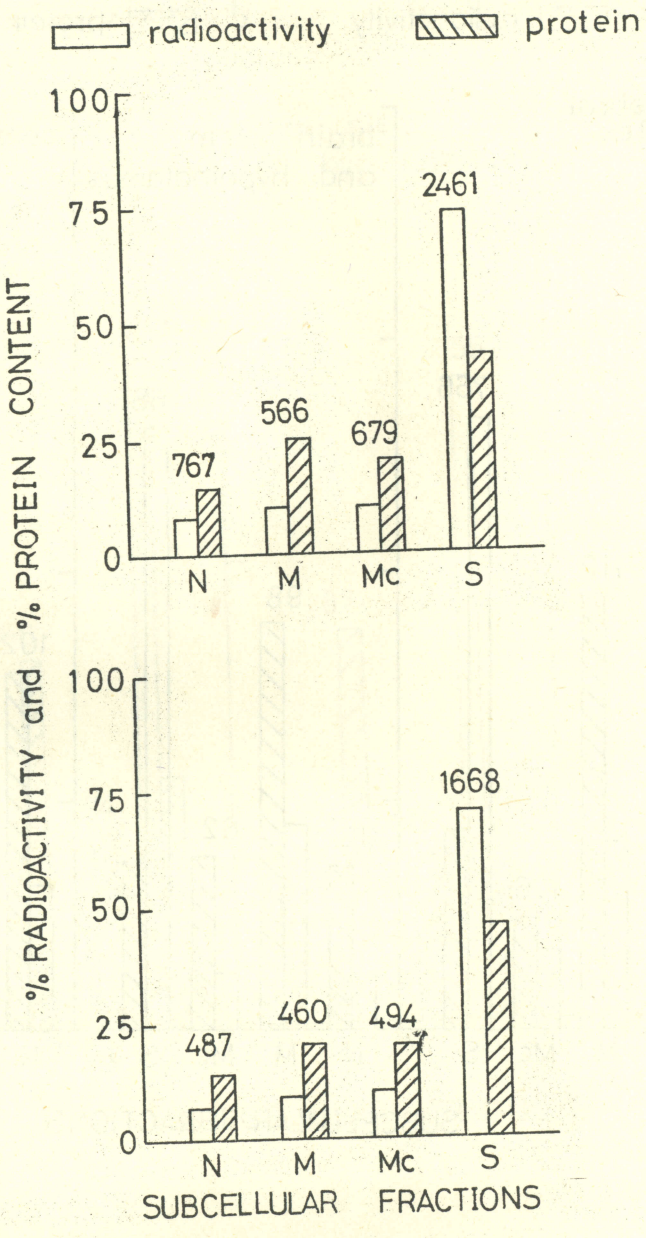

Fig. 3. Subcellular distribution patterns of radioactivity in the liver of normal mice (upper) and of naloxone treated mice (lower). N: crude nuclear fraction; M: crude mitochondrial fraction; Mc: microsomal fraction; S: soluble fraction. The numbers on the top of bars are the specific radioactivity (DPM/mg protein) of each fraction.

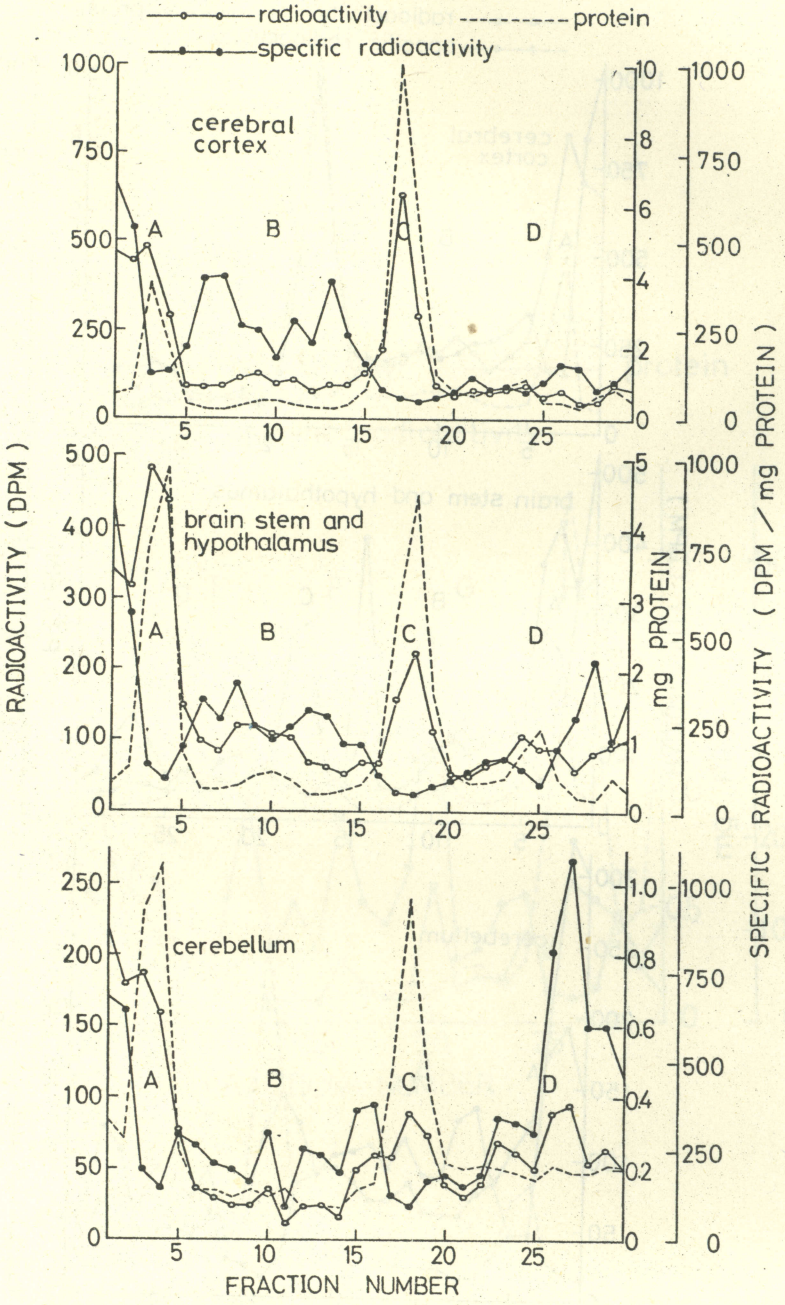

Fig. 4. Distribution of radioactivity in the components of crude mitochondrial fraction of normal mice brain tissues. A: myelin; B: heterogeneous; C: cholinergic synaptosomes; D: non-cholinergic synaptosomes.

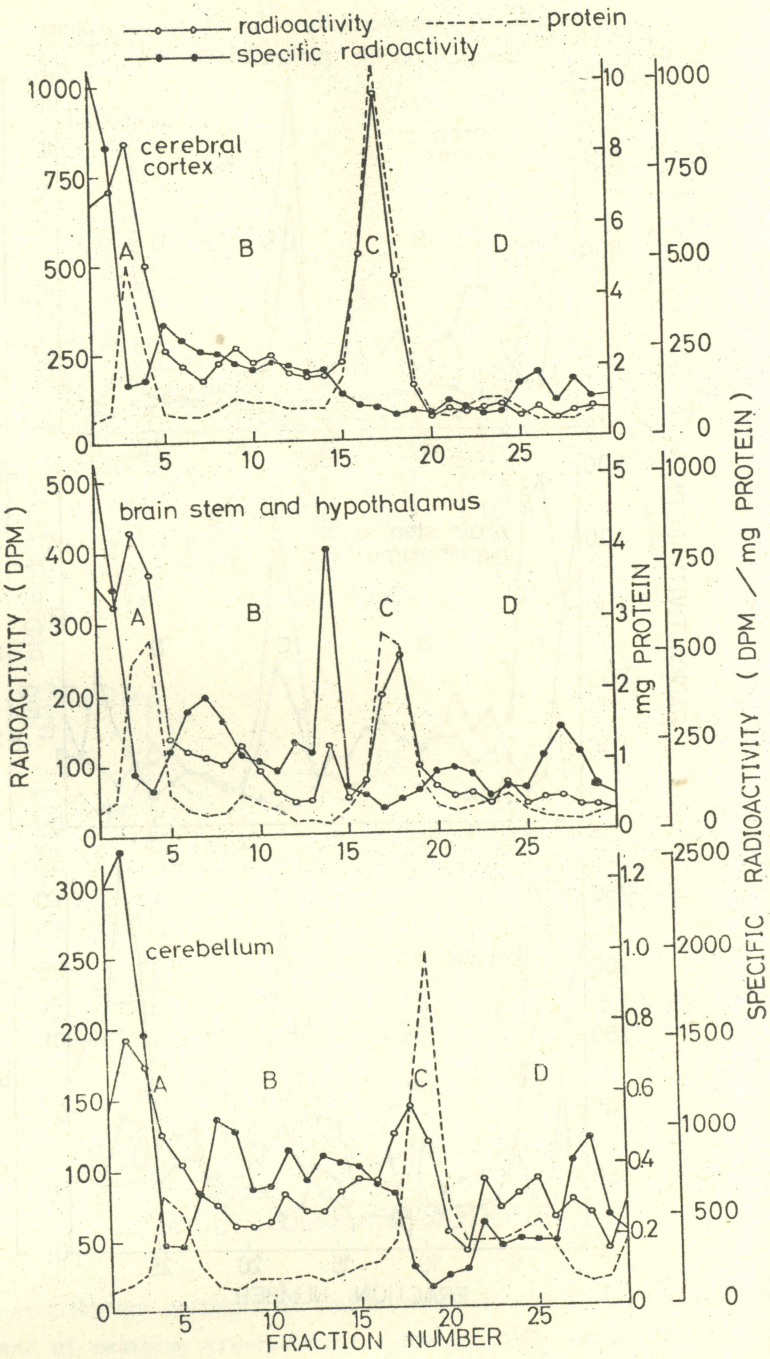

Fig. 5. Distribution of radioactivity in the components of crude mitochondrial fraction of naloxone treated mice brain tissues. A: myelin; B: heterogeneous; C: cholinergic synaptosomes; D: non-cholinergic synaptosomes.

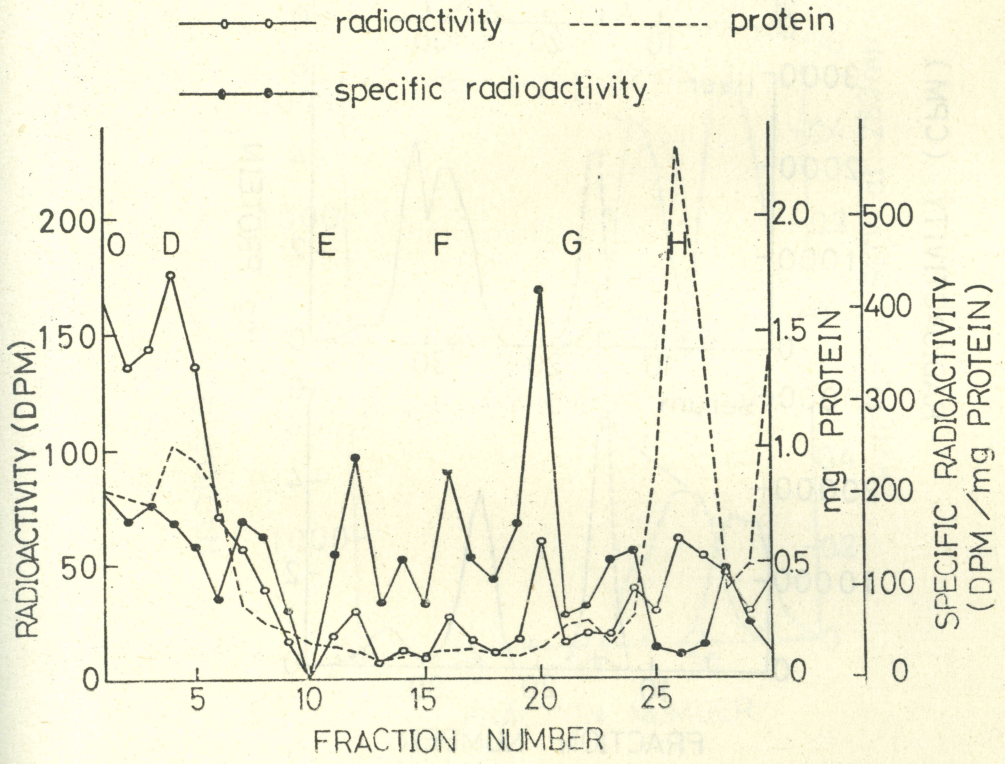

Fig. 6. Distribution of radioactivity in the components of cholinergic synaptosomes isolated from the whole brain of normal mice. O: soluble cytoplasm; D: synaptic vesicles; E: membrane; F: membrane; G: membrane; H: incompletely disrupted synaptosomes.

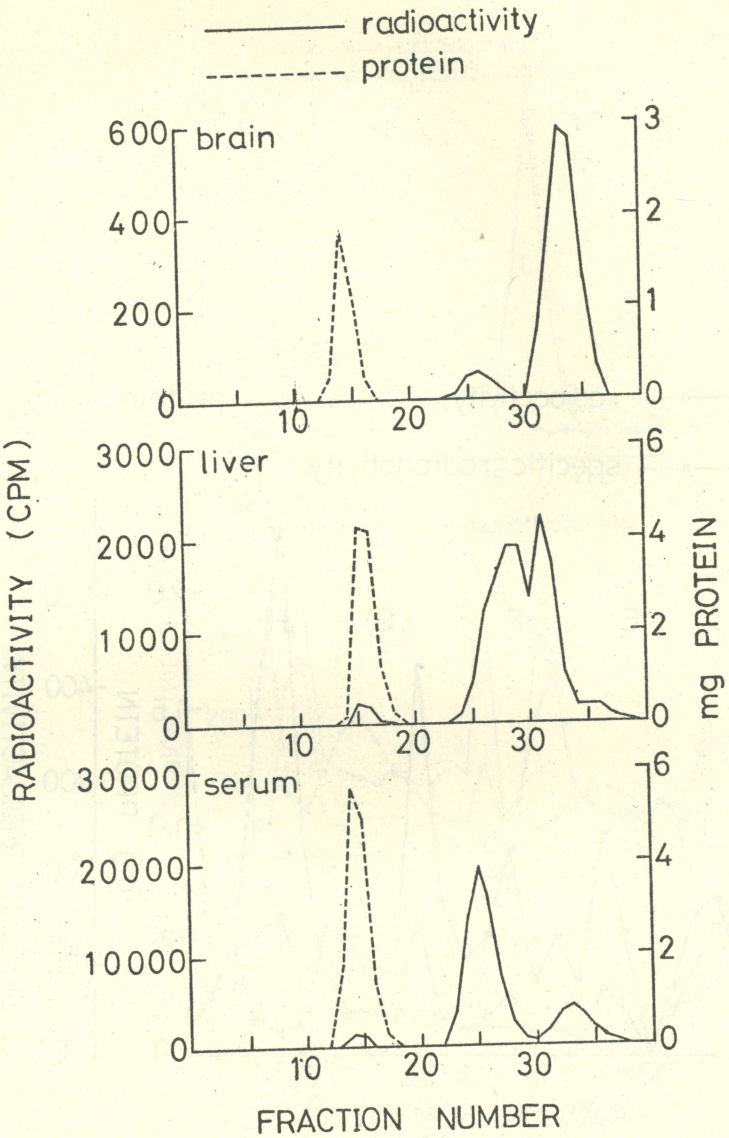

Fig. 7. Sephadex G-25 column chromatography of 1 ml brain soluble fraction, 1 ml liver soluble fraction, and 0.25 ml serum of N-methyl-c^{14} morphine injected mice.

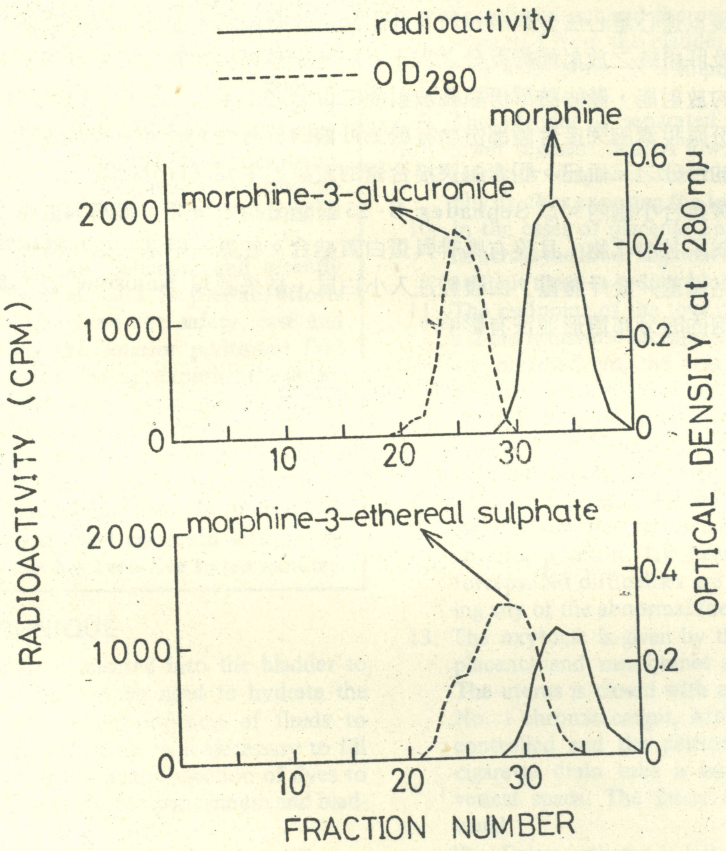

Fig. 8. Sephadex G-25 column chromatography of N-methyl-c^{14} morphine and morphine-3-monoglucuronide (upper), and of N-methyl-c^{14} morphine, morphine-3-monoglucuronide, and morphine-3-ethereal sulphate (lower).

氮—甲基—碳^{14}嗎啡在正常小白鼠及Naloxone 注射後小白鼠之腦組織與肝組織細胞內的分布

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氮—甲基—碳^{14}嗎啡經由尾靜脈注入小白鼠（每隻小白鼠注射十微居禮；劑量爲十毫克嗎啡鹽基／公斤體重），十五分鐘後以遠心離心法分離腦組織及肝組織之細胞組成，並測出放射能在此二組織細胞內之分布情形。在腦組織及肝組織之放射能約百分之六十五至七十存在於非顆粒之可溶性部份。各部份腦組織之粗腺粒體部份所含的放射能，僅佔該部份組織放射能的百分之十至二十左右。在腦組織粗腺粒體部份之組成中，僅髓鞘質部份與胆素胞突接合體部份含有較爲可觀的放射能。胆素胞突接合體部份所含的放射能，只佔腦組織放射能的百分之三而已。胆素胞突接合體的放射能主要（百分之五十五至六十）分布在它所含的可溶性原漿和胞突接合小胞內。以 Sephadex G-25 層色分析法研究，得知腦組織可溶性部份的嗎啡，僅百分之八左右變成尿甘酸化物，且沒有嗎啡與蛋白質結合。在氮—甲基—碳^{14}嗎啡注射前十五分鐘，將 Naloxone（十毫克鹽基／公斤體重）由腹腔注入小白鼠，結果發現 Naloxone 對於嗎啡進入腦組織以及放射能在此組織細胞內的分布情形並沒有影响。