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# SUBCELLULAR DISTRIBUTION OF ETORPHINE IN RAT BRAIN AND EVIDENCE FOR *in vivo* STEREOSPECIFIC BINDING

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**1** Control experiments were carried out by homogenizing rat brain at 0°C with sucrose containing various concentrations of [$^3$H]-etorphine. Subcellular fractionation of this homogenate showed that the distribution of the labelled drug amongst the primary fractions was dependent on the concentration of etorphine in the homogenate.

**2** Rats were injected intravenously with 0.2 and 20 $\mu$g/kg of [$^3$H]-etorphine. The brains were homogenized and fractionated in sucrose containing $4.2 \times 10^{-5}$ M unlabelled etorphine in order to control redistribution artifacts. Different distribution profiles in the subcellular fractions were observed at these two dose levels.

**3** Concurrent administration of either cyprenorphine or naloxone with intravenous etorphine, caused a shift of the labelled drug from the $P_3$ fraction to the supernatant fraction.

**4** The subcellular distribution of intravenously administered [$^3$H]-etorphine was also studied by homogenizing brains in etorphine-free sucrose, and sucrose containing either levorphanol or dextrorphan. From these experiments it was concluded that the $P_3$ microsomal fraction is a major site to which *in vivo* etorphine is stereospecifically bound in the rat brain.

## Introduction

Opiates, like most drugs, are believed to act by combining with specific receptors. In the past, the characterization of such receptors has proceeded by an indirect approach consisting of determination of pharmacological constants, either in isolated tissue preparations (Kosterlitz & Watt, 1968) or in intact animals (Cox & Weinstock, 1964). The notion of both agonist and antagonist potency being stereoselective for one of the enantiomers has led to the formulation of theoretical opiate receptor models (Beckett & Casy, 1954). Biochemical evidence for opiate receptors, according to the original methodology pioneered by Goldstein, Lowney & Pal (1971) was provided more recently by several investigators (Pert & Snyder, 1973; Simon, Hiller & Edelman, 1973; Terenius, 1973). There has been a growing interest in these newer studies based exclusively on *in vitro* binding techniques, which have been adopted by many other laboratories (Goldstein, 1974). However, relatively few attempts have been made to locate opiates at specific sites *in vivo*, i.e. in the only condition suitable for establishing direct correlations between drug levels and pharmacological effects.

Recently we have given preliminary accounts of experiments in which we determined the *in vivo* concentration of the highly potent morphine-like agent etorphine, in the CNS of rats. We also tentatively indicated the functional significance of these *in vivo* drug measurements (Cerletti, Manara & Mennini, 1974; Manara, Cerletti & Mennini, 1975; Manara, Aldinio, Cerletti, Coccia, Luini & Serra, 1978). This paper contains the results of studies on the subcellular distribution in rat brain of [$^3$H]-etorphine given by the intravenous route.

## Methods

### *Animals*

Male Sprague Dawley rats weighing 170–180 g were housed 6 to a plastic cage (38 × 22 × 15 cm), at constant temperature (22°C) and relative humidity (60%), with free access to water and standard food (Altromin).

Animals were killed by rapid decapitation, the brains were quickly removed and immediately placed in sucrose maintained at 0°C for the preparation of subcellular fractions.

*Preparation of subcellular fractions*

Brain primary fractions were prepared according to the basic methodology developed by De Robertis, Pellegrino de Iraldi, Rodriguez de Lores Arnais & Salganicoff (1962) and Whittaker, Michaelson & Kirkland, (1964), as previously described in detail (Manara, Carminati & Mennini, 1972a; Manara, Mennini & Cerletti, 1974). After isotonic homogenization (0.32 M sucrose) and differential centrifugation, the pellets obtained at 1,400, 11,500 and 100,000 *g* represented respectively the nuclear ($P_1$), mitochondrial ($P_2$) and microsomal ($P_3$) fractions, while the soluble fraction (S) consisted of the high speed (100,000 *g*) supernatant.

In some studies, non-radiolabelled (unlabelled) etorphine, naloxone, dextrorphan and levorphanol, at different concentrations as specified in the legends to the figures for each experiment, were added to the sucrose used for homogenization as well as for washing and resuspending the pellets.

The protein content (Lowry, Rosebrough, Farr & Randall, 1951) of the different subcellular fractions and distribution of the biochemical markers lactic dehydrogenase (LDH, Johnson, 1960) and monoamine oxidase (MAO, Bareggi, Carminati & Manara, 1971) were determined routinely on occasional samples; typical data obtained after fractionation in drug-free sucrose of brains of naive animals are shown in Table 1.

Protein and marker enzyme assay of fractions prepared either from brains of drug-treated rats and/or when drug-containing sucrose was used for fractionation gave similar findings (Aldinio, 1976).

*[$^3H$]-etorphine assay*

The pellets (fractions $P_1$, $P_2$, $P_3$), resuspended with distilled water up to 3.5 ml, and a 3.5 ml aliquot of the 100,000 *g* supernatant (fraction S) were frozen and kept at −20°C until analysed. After thawing, these specimens were mixed with a suitable quantity of unlabelled etorphine to obtain a total of about 70 μg per sample and were then extracted with 25 ml of benzene. Aliquots of benzene, transferred to egg-shaped glass containers, were evaporated in a stream of nitrogen. Dried material was redissolved in 100 μl of ethanol and aliquots for thin layer chromatography (t.l.c.) were applied to individual silica gel coated glass rods (Manara, Mennini & Carminati, 1972b) and developed in a solvent system consisting of butanol:acetic acid:distilled water (60:15:25). The silica gel in the light-yellow band of etorphine 'carrier' with $R_F = 0.70$ (resulting from degradation products which develop on exposure to environmental light and atmosphere for 18 to 24 h) was then scraped into a liquid scintillation vial containing a dioxane-based scintillation solution (Manara *et al.*, 1972b). This procedure results in complete elution of the drug, which can then be counted in solution without any interference from the silica gel, which settles on the bottom of the vial.

The counting instrument was a Packard Tri Carb model C 2425, operated at 14°C. Samples were counted to a statistical error not exceeding ±6%. Counting efficiency, determined by internal standardization on randomly chosen samples and monitored on all the samples with the counter's own automatic external standardization device, averaged 42%. Unlabelled samples, processed in the same way as the radioactive samples had a counting rate comparable to the background, which ranged between 15 and 20 counts per minute as determined on the entire stock of counting vials before adding the radioactive aliquots. Analysis of the brains to which [$^3$H]-etorphine

**Table 1** Lactic dehydrogenase (LDH), monoamine oxidase (MAO) and protein content of brain subcellular fractions from naive rats

| *Fractions* | *LDH* (μmol $min^{-1}$ $g^{-1}$) | *MAO* (μmol $min^{-1}$ $g^{-1}$) | *Protein* (mg/g) |
|---|---|---|---|
| $P_1$ | 3.27 ± 0.90 | 37.2 ± 0.7 | 23.3 ± 1.0 |
| $P_2$ | 6.87 ± 1.95 | 66.4 ± 2.9 | 30.8 ± 3.6 |
| $P_3$ | 2.49 ± 0.15 | 10.7 ± 5.6 | 36.5 ± 0.6 |
| S | 17.19 ± 0.99 | 9.6 ± 1.0 | 55.4 ± 1.8 |

Figures are mean values ± s.d., each based on 3 determinations, and refer to the total material recovered per fraction after processing 1 g of brain tissue. The values (μmol $min^{-1}$ $g^{-1}$) for LDH and MAO activity indicate the rate of substrate disappearance and product formation respectively, i.e. NADH and 4-hydroxyquinoline in that order. Drug-free sucrose was used throughout the fractionation process.

was added in the homogenizer (redistribution samples) also served to determine the assay recoveries; the sum of [$^3$H]-etorphine activities found in the chromatograms of the different fractions of each brain accounted for 102.1 ± 1.6 (mean ± s.d., $n$ = 15) of the labelled etorphine added initially. Recoveries were similar when [$^3$H]-etorphine was added directly to any of the fractions. Redistribution samples were included in each experiment, even when not reported in the figures, and proved highly reproducible.

*Experimental design and statistical analysis*

The data discussed in the results section were obtained after 3 or 4 replications (specified for each study) on different days in each of the experimental conditions. The daily design, included an equal number of samples for each experimental condition. The fractions from each brain were assayed individually and percentage values of [$^3$H]-etorphine calculated (the sum of [$^3$H]-etorphine in the 4 fractions from each brain was taken as 100%).

The original data were then evaluated by a two-factor measures split-plot design (3 × 4) (Kirk, 1968) after transformation into $x' = 2 \arcsin \sqrt{x}$, where x is expressed as a proportion. The significance of the differences between means compared one with another, in pairs, was assessed by Tukey's multiple comparison HSD (honestly significant difference) test (Tukey, 1953).

*Drugs*

The following drugs, gifts of which are gratefully acknowledged, were freshly dissolved in 0.9% w/v NaCl solution (saline) and administered either intravenously (tail vein), subcutaneously or intraperitoneally as specified for each experiment in the legend to the appropriate figure.

[$^3$H]-etorphine hydrochloride, labelled in the 15 and 16 positions, sp. act. 28 Ci/mM, was found to be over 70% radiochemically pure in our laboratory; etorphine hydrochloride; cyprenorphine hydrochloride (Reckitt & Colman); naloxone hydrochloride (Endo Laboratories Inc., Garden City, N.Y., U.S.A.); Dextrorphan (+)-tartrate monohydrate and levorphanol (−)-tartrate dihydrate (Dr D. Della Bella, Zambon S.p.A., Milan, Italy) were also used.

## Results

*Control experiments*

The apparent *in vivo* subcellular distribution profile of a drug obtained by cell fractionation techniques may be significantly affected by redistribution artifacts arising in the course of the fractionation (Manara *et al.*, 1974). To ascertain whether similar artifacts are involved in the case of [$^3$H]-etorphine, we added this drug to the homogenizer containing the brain tissue from untreated rats and determined the etorphine pattern in the subcellular fractions. As shown in Figure 1, [$^3$H]-etorphine is redistributed from the soluble to the particulate fractions to a varying extent, depending on the total drug concentration in the system. The concentrations of $3.1 \times 10^{-11}$ and $2.1 \times 10^{-9}$ M encompass the levels found in rat brain 15 min after administration of a threshold dose of etorphine (0.2 μg/kg i.v.) i.e. the analgesic $ED_{50}$ (Blane, Boura, Fitzgerald & Lister, 1967) and of a large dose (20 μg/kg i.v., $>ED_{100}$ for producing loss of righting reflex) respectively. At etorphine concentrations above $2.1 \times 10^{-9}$ M the drug was found predominantly in the supernatant fraction, whereas a differential distribution was clearly evident at the lower concentrations of etorphine. It was also apparent that there was a considerable variation in the distribution

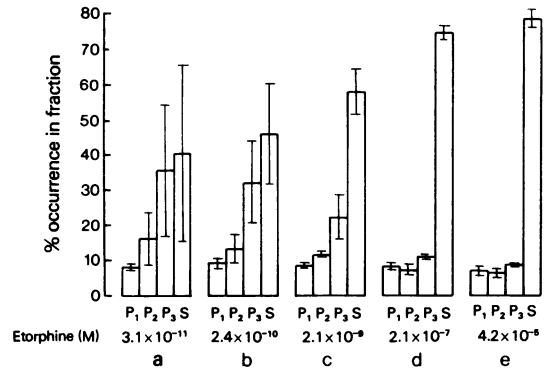


**Figure 1** Distribution in rat brain subcellular fractions of [$^3$H]-etorphine added in the homogenizer: dependence on etorphine concentration. The columns indicate mean values, each based on 3 determinations and representing the percentage of [$^3$H]-etorphine activity found in each fraction (the sum of [$^3$H]-etorphine activities of the four fractions prepared from the same brain was made equal to 100%). Vertical lines show s.d. The molar concentration of etorphine added in the homogenizing sucrose is specified under each histogram. For comparison of fractions of the same type in different experimental conditions, statistical analysis (see Methods) showed significant differences: for $P_3$, a ≠ e, a ≠ d, b ≠ e, b ≠ d; for S, a ≠ e, a ≠ d, b ≠ e, b ≠ d, ($P < 0.01$). Comparison of the different fractions under one particular experimental condition showed: in (a) and (b), $P_2 \neq P_3$ ($P < 0.05$), $P_1 \neq P_3$, $P_1 \neq S$, $P_2 \neq S$ ($P < 0.01$); in (c), $P_1 \neq P_3$, $P_2 \neq S$ ($P < 0.05$), $P_1 \neq S$, $P_3 \neq S$ ($P < 0.01$); in (d) and (e), $P_1 \neq S$, $P_2 \neq S$, $P_3 \neq S$ ($P < 0.01$).

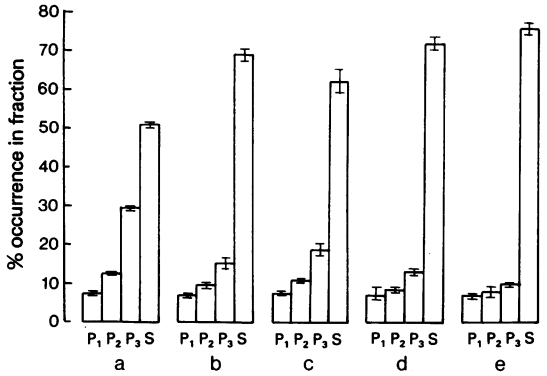


**Figure 2** Subcellular distribution of [$^3$H]-etorphine in rat brain: dependence on injected dose and on antagonist pretreatment. The columns indicate mean values ($n = 4$) of the percentage of [$^3$H]-etorphine found in each fraction. Vertical lines show s.d. Each histogram refers to a specific experimental condition as shown by the letter below. (a and c): [$^3$H]-etorphine hydrochloride was injected (i.v.) to rats at doses of 0.2 and 20 μg/kg respectively. (b and d): [$^3$H]-etorphine hydrochloride (0.2 and 20 μg/kg i.v. respectively) was administered to cyprenorphine-pretreated rats (100 μg/kg, i.p. 30 min before etorphine). All the drug-treated animals were killed 15 min after [$^3$H]-etorphine administration. (e): [$^3$H]-etorphine was added to brain tissue in the homogenizer from drug-free animals. Tissue fractionation was performed in sucrose containing $4.2 \times 10^{-5}$ M unlabelled etorphine. Total brain concentrations of [$^3$H]-etorphine hydrochloride were $96 \pm 6$ and $71 \pm 5$ pg/g (mean ± s.d.) in experimental conditions (a) and (b) respectively; $13.2 \pm 4.0$ and $6.2 \pm 0.3$ ng/g (mean ± s.d.) in experimental conditions (c) and (d) respectively. For comparison of fractions of the same type in different experimental conditions, statistical analysis (see Methods section) showed the following significant differences: for $P_2$, a ≠ b, a ≠ d, a ≠ e, c ≠ d, c ≠ e ($P < 0.01$) for $P_3$ and S, all the conditions, except b versus d, different at $P < 0.01$. A comparison of the different fractions under one particular experimental condition showed that: in (a), (b) and (c) all the values were significantly different from each other at the 0.01 level; in (d) and (e) all the values, except those of fractions $P_1$ and $P_2$, were significantly different from each other ($P < 0.01$).

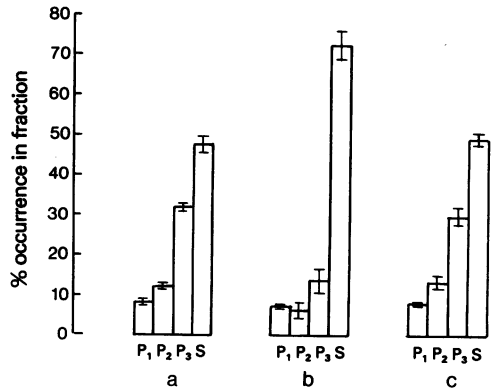


**Figure 3** Subcellular distribution of [$^3$H]-etorphine in rat brain: effect of antagonist pretreatment and of adding antagonist in the homogenizer. The columns indicate mean values ($n = 3$) of the percentage [$^3$H]-etorphine found in each fraction. Vertical lines show s.d. [$^3$H]-etorphine hydrochloride (0.2 μg/kg i.v.) was given to control (a and c) or naloxone-pretreated (450 μg/kg, s.c., 30 min before) rats (b). The animals were all killed 15 min after etorphine administration. Tissue fractionation was performed in sucrose containing either etorphine ($4.2 \times 10^{-5}$ M) alone (a and b) or etorphine plus $2.6 \times 10^{-4}$ M naloxone (c). Total brain concentrations of [$^3$H]-etorphine hydrochloride were: $0.14 \pm 0.03$; $0.07 \pm 0.01$; $0.12 \pm 0.002$ ng/g (mean ± s.d.) in (a), (b) and (c) respectively. For a comparison of fractions of the same type in different experimental conditions, statistical analysis (see Methods section) showed significant differences for $P_2$, $P_3$ and S: a ≠ b and b ≠ c ($P < 0.01$). A comparison of the different fractions under one particular experimental condition showed that all the values were significantly different from each other at the 0.01 level, except for the differences between $P_1$ and $P_2$ in condition (a) (significant at $P < 0.05$) and between $P_1$ and $P_2$ in condition (b) (not significantly different).

of [$^3$H]-etorphine at the lower drug concentrations, as shown by the large standard deviations in Figure 1.

*Subcellular distribution after* in vivo *labelling*

In view of the above results, the following experiments were performed in which $4.2 \times 10^{-5}$ M unlabelled etorphine was added to the homogenizing medium in order to control redistribution artifacts. Figure 2 shows the distribution of [$^3$H]-etorphine under different conditions. The subcellular distribution patterns following injection of 0.2 and 20 μg/kg labelled etorphine (Figure 2a and c respectively) were significantly different from each other, particularly as regards the relative concentrations in fractions $P_3$ and S. The profiles (a) and (c) obtained after administration of [$^3$H]-etorphine *in vivo*, also differed from profile (e), which was obtained by addition of the labelled drug to untreated brain tissue in the homogenizer.

The influence of the concurrent administration to the animal of an opiate antagonist, cyprenorphine, on the subcellular distribution of [$^3$H]-etorphine in the brain is apparent from Figure 2(b) and (d) which

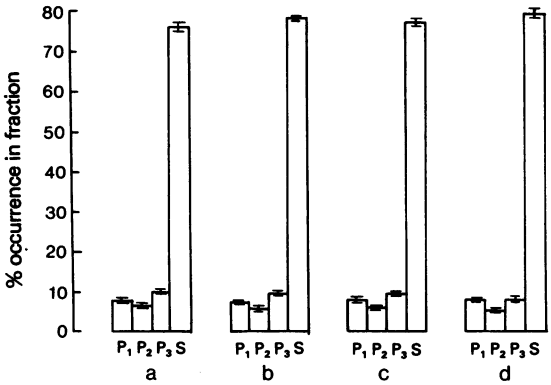


**Figure 4** Distribution of [$^3$H]-etorphine added in the homogenizer in rat brain subcellular fractions: effect of antagonist pretreatment and of adding antagonist in the homogenizer. The columns indicate mean values ($n$ = 3) of the percentage of [$^3$H]-etorphine found in each fraction. Vertical lines show s.d. [$^3$H]-etorphine was added in the homogenizer to brain tissue from naive rats, except for condition (b), in which the brains were obtained from naloxone-treated animals (860 μg/kg s.c., 45 min before being killed). Tissue fractionation was performed in sucrose containing either etorphine alone $4.2 \times 10^{-5}$ M, (a) and (b), or etorphine plus naloxone $5.1 \times 10^{-9}$ M or $2.6 \times 10^{-4}$ M, (c) and (d) respectively. For a comparison of fractions of the same type each in different experimental conditions, statistical analysis (see Methods section) showed significant differences for $P_3$ and S only between conditions (a) and (d) ($P < 0.05$). A comparison of the different fractions under one particular experimental condition showed all the values to be significantly different from each other at least at the 0.01 level, except for the differences between $P_1$ and $P_2$ in condition (a) (significant at $P < 0.05$) and between $P_1$ and $P_3$ in condition (c) (not significantly different).

significantly differ from the respective control profiles (a) and (c). The main effect of the antagonist was to cause a shift of the drug from the $P_3$ to the S fraction (compare a and b of Figure 2). Further experiments were performed to determine whether this effect depends on the concurrently administered narcotic antagonist interacting *in vivo* with the tissue binding of [$^3$H]-etorphine, or on an *in vitro* mechanism (i.e. during homogenization).

The data in Figure 3 show that when naloxone is administered to the animals concurrently with labelled etorphine, then, as previously seen for cyprenorphine, the distribution is different from that obtained following fractionation of the brains of rats given [$^3$H]-etorphine alone (compare a and b in Figure 3). Conversely, the addition of naloxone in the homogenizer under the test conditions (i.e. using sucrose containing unlabelled etorphine), has no apparent effect on the percentage distribution in the different fractions of tritiated etorphine given to the rat (compare a and c, Figure 3). Moreover, as shown in Figure 4, naloxone did not alter the distribution of [$^3$H]-etorphine that was added directly to the homogenizer containing unlabelled brain tissue. Thus the 'control' distribution of [$^3$H]-etorphine was unaffected by previously treating the animal with naloxone (Figure 4b) or by addition of naloxone up to $2.6 \times 10^{-4}$ M to the homogenizer (Figure 4c and d).

*Distribution of [$^3$H]-etorphine in carrier-free media*

Homogenization of brains of rats treated with [$^3$H]-etorphine, in etorphine-free sucrose, gave subcellular distribution patterns (Figure 5a) significantly different from those of similar brains homogenized in sucrose containing unlabelled etorphine (Figure 5b and c). Figure 5(a) shows a peak concentration of [$^3$H]-etorphine in the microsomal fraction ($P_3$); addition to the homogenizing medium of even moderate concentrations of unlabelled etorphine ($4.2 \times 10^{-8}$ M, Figure 5b) reduces the particulate bound ($P_3$) labelled drug, therefore increasing its occurrence in the supernatant.

The same effect on particulate bound [$^3$H]-etorphine from brains of rats treated with the labelled drug *in vivo* can be elicited by addition to the homogenizing medium of other opiates such as levorphanol, as well as narcotic antagonists, which are not shown (compare Figure 6, a and b). Further, the activity of the opiate added to sucrose appears to depend on its stereochemical structure. Dextrophan was virtually inactive at a concentration comparable to that at which its enantiomer, levorphanol, produced significant changes in the control profile, and even after a 100-fold increase in concentration, dextrorphan still failed to produce effects comparable to those of levorphanol (see Figure 6).

## Discussion

For a critical appraisal of the apparent subcellular distribution in rat brain of [$^3$H]-etorphine it was necessary to perform control experiments by addition of [$^3$H]-etorphine to cerebral tissue from drug-free animals at 0°C, before homogenization and separation. The *in vitro* distribution pattern probably result from physico-chemical factors (e.g. partition coefficient). Surface phenomena are the most likely explanation for the poor reproducibility of the distributions observed at the lowest drug concentrations and may also account for the finding that up to $2.1 \times 10^{-7}$ M etorphine the percentage of the drug

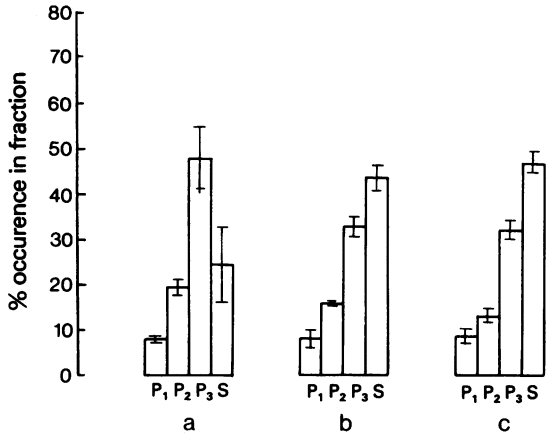


**Figure 5** Subcellular distribution of $^3$H-etorphine in rat brain obtained by homogenization in 'carrier'-free or in homogenizing medium containing unlabelled etorphine. The column indicate mean values ($n = 3$) of the percentage of [$^3$H]-etorphine found in each fraction. Vertical lines show s.d. All the animals were injected with [$^3$H]-etorphine hydrochloride, (0.2 μg/kg i.v.) 15 min before being killed. Tissue fractionation was performed either in etorphine-free sucrose (a) or in sucrose containing $4.2 \times 10^{-8}$ or $4.2 \times 10^{-6}$ M etorphine (b and c respectively). Total brain concentrations of [$^3$H]-etorphine hydrochloride were: 0.11 ± 0.02; 0.12 ± 0.02; 0.12 ± 0.01 ng/g (mean ± s.d.) in (a), (b) and (c) respectively. For a comparison of fractions of the same type in different experimental conditions, statistical analysis (see Methods section) showed the following significant differences at $P < 0.01$ for $P_3$ and S: a ≠ b and a ≠ c. A comparison of the different fractions under one particular experimental condition, showed that all the values were significantly different from each other at least at the 0.01 level, except for the differences between $P_2$ and S in condition (a) and between $P_1$ and $P_2$ in condition (c) (not significantly different).

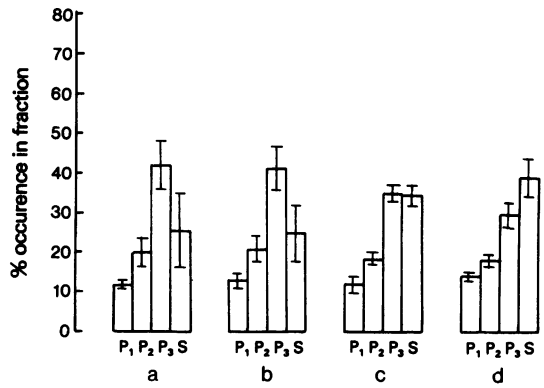


**Figure 6** Subcellular distribution of [$^3$H]-etorphine in rat brain: dependence on stereochemical configuration of opiate 'carrier' added to homogenizing medium. The columns indicate mean values ($n = 4$) of the percentage of [$^3$H]-etorphine found in each fraction. Vertical lines show s.d. All the animals were injected with [$^3$H]-etorphine hydrochloride (0.2 μg/kg i.v.) 15 min before being killed. Tissue fractionation was performed either in 'carrier'-free sucrose (a), or in sucrose containing dextrophan ($4.4 \times 10^{-7}$ or $4.4 \times 10^{-5}$ M, b and c respectively) or $4.2 \times 10^{-7}$ M levorphanol (d). Total brain concentrations of [$^3$H]-etorphine hydrochloride were: 0.13 ± 0.02; 0.11 ± 0.01; 0.12 ± 0.02; 0.11 ± 0.01 ng/g (mean ± s.d.) in (a), (b), (c) and (d), respectively. For a comparison of fractions of the same type in different experimental conditions, statistical analysis (see Methods section) showed the following significant differences: for $P_3$, a ≠ d, b ≠ d ($P < 0.01$) for S, a ≠ d, b ≠ d ($P < 0.01$) and a ≠ c, b ≠ c ($P < 0.05$). Comparison of the different fractions under one particular experimental condition gave the following differences in (a) and (b), $P_1 \neq P_3$, $P_2 \neq P_3$, $P_3 \neq S$, $P_1 \neq S$ ($P < 0.01$), $P_1 \neq P_2$ ($P < 0.05$); in (c), $P_1 \neq P_3$, $P_2 \neq P_3$, $P_1 \neq S$, $P_2 \neq S$ ($P < 0.01$); in (d) $P_1 \neq S$, $P_2 \neq S$, $P_1 \neq P_3$ ($P < 0.01$), $P_2 \neq P_3$ ($P < 0.05$).

found in the different fractions depends on the concentration of drug used.

These *in vitro* results counsel caution in interpreting the apparent subcellular distribution profile of etorphine obtained after fractionation of brains from animals receiving this drug *in vivo*. The percentage of the drug found in a given fraction may in fact mostly reflect *in vitro* redistribution, obscuring the original *in vivo* drug binding to the subcellular components isolated in that fraction. Therefore our safest conclusions appear to be based on a comparison of the subcellular distribution profiles as a whole, obtained by operating with a constant concentration of unlabelled etorphine 'carrier' in the homogenizing sucrose.

Under such assay conditions the differences between the subcellular distribution profiles are assumed to denote a differential drug binding in the brain, regardless of the location of the binding sites or their occurrence in any given fraction, both of which remain to be established. Typically, such differences are evident in comparing the profiles obtained after administering a threshold or a 100-fold pharmacological dose of etorphine as shown in Figure 2. Similar differences are apparent between the profiles from control animals receiving etorphine alone and from animals treated concurrently with an opiate antagonist (Figures 2 and 3) and the results suggest that the antagonist affects etorphine binding *in vivo* (Figure 3).

From our data it may be concluded that the lowered brain etorphine concentration in cases of concurrent administration of an opiate antagonist (Cerletti *et al.*, 1974; Manara *et al.*, 1975), depends at least in part on altered binding of the drug in the brain.

Following the administration of a threshold pharmacological dose of tritiated etorphine (0.2 μg/kg) and homogenization of the brain in sucrose containing no etorphine, the labelled drug was found to be concentrated in the microsomal ($P_3$) fraction. The profile of subcellular distribution obtained in this *in vivo* condition, was far more reproducible than that obtained by adding a corresponding amount of labelled etorphine to cerebral tissue from untreated rats, in the homogenizer at 0°C. It should be pointed out that the distribution of [$^3$H]-etorphine in the different subcellular fractions when added to the homogenizer, does not account for the prevailing localization of the labelled drug in the $P_3$ fraction after *in vivo* administration of [$^3$H]-etorphine.

Our results suggest that the microsomal fraction may contain a major site for the *in vivo* binding of etorphine. An insight into some features of this binding was provided by additional experiments. The radiolabelled etorphine bound to the microsomal fraction after *in vivo* administration, was released to a significant extent by adding unlabelled etorphine to the homogenizing sucrose. The concentrations of the labelled and unlabelled drug to which this finding refers, are respectively lower and higher than the concentration of etorphine ($2 \times 10^{-9}$ M) which saturates opiate receptor stereospecific binding in *in vitro* studies (Simon *et al.*, 1973). The ability of an opiate in the homogenizing sucrose to release *in vivo* administered labelled etorphine from the microsomal fraction, was also stereospecific. Therefore [$^3$H]-etorphine binding to the microsomal fraction following *in vivo* administration, under our conditions appears to reflect in part a mechanism involving stereospecificity.

We believe these results are important because the *in vivo* stereospecific binding we have found in the microsomal fraction, involves a significant percentage (at least 15%) of the total drug present in the brain. However, more refined fractionation techniques are required to isolate the precise subcellular structures containing the stereospecific binding sites. In previous studies by other investigators, administering less powerful opiates than etorphine *in vivo* to laboratory animals, the brain subcellular distribution of the drugs primarily reflected non-specific factors such as the lipid solubility of each compound, rather than pharmacological effectiveness or stereo-isomerism (Mellett & Woods, 1959; Van Praag & Simon, 1966; Mulé, Redman & Flesher, 1967; Clouet & Williams, 1973; Mulé, Casella & Clouet, 1974). These negative findings are very probably due to the high total drug concentration in the brain, compared to the supposedly much smaller amounts bound to receptor sites which are too small to be unmasked.

Our results and conclusions do not agree with those of Mulé, Casella & Clouet (1975). These authors administered a relatively large amount of radiolabelled etorphine intracisternally to rats; the radioactivity recovered in whole brain homogenates gave a total drug concentration of approximately 500 times that found in our experiments (i.e. about 0.1 ng/g brain), after systemic administration of a threshold pharmacological dose of etorphine (0.2 μg/kg). Mulé *et al.* (1975) found less than 1% of the total activity in the brain, in subfractions of the osmotically shocked mitochondrial fraction. Yet these subfractions, in their opinion, contain the specific binding sites of etorphine. Furthermore, the main conclusion from the same study was that this binding is stereospecific, because the differences in binding between control and narcotic antagonist pretreated animals, was of the same magnitude as that between animals pretreated either with (+)-cyclorphan (inactive isomer) or (−)-cyclorphan (active antagonist). However, etorphine was bound to a similar extent in control and in (+)-cyclorphan pretreated rats, which leads us to disagree with the authors' conclusions. Pert & Snyder (1973) concluded from *in vitro* studies that the largest number of stereospecific opiate binding sites is in the crude mitochondrial fraction and its subfractions (Pert, Snowman, & Snyder, 1974). This is not easy to reconcile with our data (Figures 5 and 6) and our conclusions that the microsomal fraction contains most of the material to which etorphine is bound stereospecifically *in vivo*. However, our results also indicate that the microsomal fraction is richest in *in vivo* stereospecifically bound etorphine (drug bound per mg protein). In this respect our findings are to some extent in agreement with both the data of the aforementioned *in vitro* binding studies (Pert & Snyder, 1973; Pert, Snowman, & Snyder, 1974) and more recent results, also obtained by *in vitro* assay on rat striatum (Leysen & Laduron, 1977) or mouse brain (Smith & Loh, 1976) homogenates.

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## References


ALDINIO, C. (1976). Analisi degli indici biochimici lattico-deidrogenasi e monoaminossidasi come parametri di riferimento nello studio della distribuzione subcellulare di un farmaco nel cervello di ratto. *University of Milan, Doctoral Thesis.*

BAREGGI, S.R., CARMINATI, P. & MANARA, L. (1971). Metaraminol and monoamine oxidase activity in the rat heart. *Res. Commun. Chem. Path. Pharmac.*, **2**, 347–354.

BECKETT, A.H. & CASY, A.F. (1954). Synthetic analgesics: Stereochemical considerations. *J. Pharm. Pharmac.*, **6**, 986–999.

BLANE, G.F., BOURA, A.L.A., FITZERALD, A.E. & LISTER, R.E. (1967). Actions of etorphine hydrochloride (M99): A potent morphine-like agent. *Br. J. Pharmac. Chemother.*, **30**, 11–22.

CERLETTI, C., MANARA, L. & MENNINI, T. (1974). Brain levels of the potent analgesic etorphine in rats and their functional significance. *Br. J. Pharmac.*, **52**, 440–441P.

CLOUET, D.H. & WILLIAMS, N. (1973). Localization in brain particulate fractions of narcotic analgesic drugs administered intracisternally to rats. *Biochem. Pharmac.*, **22**, 1283–1293.

COX, B.M. & WEINSTOCK, M. (1964). Quantitative studies of the antagonism by nalorphine of some of the actions of morphine-like analgesic drugs. *Br. J. Pharmac. Chemother.*, **22**, 289–300.

DE ROBERTIS, E., PELLEGRINO de IRALDI, A., RODRIGUEZ DE LORES ARNAIZ, G. & SALGANICOFF, L. (1962). Cholinergic and non-cholinergic nerve endings in rat brain. I. Isolation and subcellular distribution of acetylcholine and acetylcholinesterase. *J. Neurochem.*, **9**, 23–35.

GOLDSTEIN, A. (1974). Minireview. Opiate receptors. *Life Sci.*, **14**, 615–623.

GOLDSTEIN, A., LOWNEY, L.I. & PAL, B.K. (1971). Stereospecific and nonspecific interactions of the morphine congener levorphanol in subcellular fractions of mouse brain. *Proc. natn. Acad. Sci. USA*, **68**, 1742–1747.

JOHNSON, M.K. (1960). The intracellular distribution of glycolytic and other enzymes in rat-brain homogenates and mitochondrial preparations. *Biochem. J.*, **77**, 610–618.

KIRK, R.E. (1968). Experimental Design: Procedures for the Behavioral Sciences. Belmont, California: Brooks/Cole Publ. Co.

KOSTERLITZ, H.W. & WATT, A.J. (1968). Kinetic parameters of narcotic agonists and antagonists, with particular reference to N-allylnoroxymorphone (Naloxone). *Br. J. Pharmac. Chemother.*, **33**, 266–276.

LEYSEN, J. & LADURON, P. (1977). Differential distribution of opiate and neuroleptic receptors and dopamine-sensitive adenylate cyclase in rat brain. *Life Sci.*, **20**, 281–288.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265–275.

MANARA, L., ALDINIO, C., CERLETTI, C., COCCIA, P., LUINI, A. & SERRA, G. (1978). *In vivo* tissue levels and subcellular distribution of opiates with reference to pharmacological action. In *Factors Affecting the Action of Narcotics.* ed. Adler, M.W., Manara, L. & Samanin, R. New York: Raven Press, (in press).

MANARA, L., CARMINATI, P. & MINNINI, T. (1972a). "*In vivo*" persistent binding of $^3$H-reserpine to rat brain subcellular components. *Eur. J. Pharmac.*, **20**, 109–113.

MANARA, L., CERLETTI, C. & MENNINI, T. (1975). Etorphine (ET): *In vivo* measurements and significance. *Fedn Proc.*, **34**, abst. 3367.

MANARA, L., MENNINI, T. & CARMINATI, P. (1972b). Reduced binding of $^3$H-reserpine to hearts of 6-hydroxydopamine-pretreated rats. *Eur. J. Pharmac.*, **17**, 183–185.

MANARA, L., MENNINI, T. & CERLETTI, C. (1974). $^3$H-reserpine persistently bound "*in vivo*" to rat brain subcellular components: Limited removal by peanut oil extraction. *Life Sci.*, **14**, 2267–2276.

MELLETT, L.B. & WOODS, L.A. (1959). The intracellular distribution of N-C$^{14}$-methyl levorphanol in brain, liver and kidney tissue of the rat. *J. Pharmac. exp. Ther.*, **125**, 97–104.

MULÉ, S.J., CASELLA, G. & CLOUET, D.H. (1974). Localization of narcotic analgesics in synaptic membranes of rat brain. *Res. Commun. Chem. Path. Pharmac.*, **9**, 55–77.

MULÉ, S.J., CASELLA, G. & CLOUET, D.H. (1975). The specificity of binding of the narcotic agonist etorphine in synaptic membranes of rat brain *in vivo*. *Psychopharmacologia*, **44**, 125–129.

MULÉ, S.J., REDMAN, C.M. & FLESHER, J.W. (1967). Intracellular disposition of H$^3$-morphine in the brain and liver of nontolerant and tolerant guinea pigs. *J. Pharmac. exp. Ther.*, **157**, 459–471.

PERT, C.B., SNOWMAN, A.M. & SNYDER S.H. (1974). Localization of opiate receptor binding in synaptic membranes of rat brain. *Brain Res.*, **70**, 184–188.

PERT, C.B. & SNYDER, S.H. (1973). Opiate receptor: Demonstration in nervous tissue. *Science*, **179**, 1011–1014.

SIMON, E.J., HILLER, J.M. & EDELMAN, I. (1973). Stereospecific binding of the potent narcotic analgesic [$^3$H]-etorphine to rat-brain homogenate. *Proc. natn. Acad. Sci. USA*, **70**, 1947–1949.

SMITH, A.P. & LOH, H.H. (1976). The sub-cellular localization of stereo-specific opiate binding in mouse brain. *Res. Commun. Chem. Path. Pharmac.*, **15**, 205–219.

TERENIUS, L. (1973). Characteristics of the "receptor" for narcotic analgesics in synaptic plasma membrane fraction from rat brain. *Acta pharmac. tox.*, **33**, 377–384.

TUKEY, J.W. (1953). *The Problem of Multiple Comparisons.* Princeton University.

VAN PRAAG, D. & SIMON, E.J. (1966). Studies on the intracellular distribution and tissue binding of dihydromorphine-7,8-H$^3$ in the rat. *Proc. Soc. exp. Biol. Med.*, **122**, 6–11.

WHITTAKER, V.P., MICHAELSON, I.A., & KIRKLAND, R.J.A. (1964). The separation of synaptic vesicles from nerve-ending particles ('Synaptosomes'). *Biochem. J.*, **90**, 293–303.


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