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Multiple Molecular Forms of Stereospecific Opiate Binding

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SUMMARY

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When brain membranes were incubated *in vitro* with ^{3}H-enkephalinamide, then extracted with the non-ionic detergent Brij 36-T, up to 75% of the levorphanol-displaceable radioactivity was released in a bound form. Analysis of the binding material by gel filtration revealed a broad peak of 100,000-500,000 molecular weight, and several other species of less than 20,000 molecular weight. Iso-electric focusing resolved the binding components into two major peaks of pI's about 8.4 and 3.2, and several minor species in the pI range 8.3-6.5; both high and low molecular weight material appeared to be present in the two major pI peaks. All of the stereospecific binding components identified by iso-electric focusing appeared to behave similarly with respect to several competing unlabeled drugs, and to Na^+ ion. Comparable heterogeneity was observed in material stereospecifically binding ^{3}H-β_H-endorphin, ^{3}H-etorphine, and ^{3}H-naloxone, and incubation of Brij-extracted ^{3}H-β_H-endorphin-binding membranes with 10 mM dimethyl suberimidate covalently labeled a broad range of species of 2-200 $\times$ 10^3 molecular weight. These results demonstrate that many distinct brain membrane components can bind opiates stereospecifically *in vitro*; these components may include lipids as well as proteins.

INTRODUCTION

Several years ago, it was demonstrated that brain membrane fractions can bind opiates stereospecifically *in vitro*, and some evidence suggests that this binding is to the pharmacologically-relevant receptor (1-3). To date, little information about the membrane component responsible for binding has accumulated, because of its resistance to solubilization in an active form; however, an important advance was made by Simon *et al.* (4), who succeeded in extracting an opiate-protein complex from brain membranes with the non-ionic detergent Brij 36-T (poly(oxyethylene) 10-lauryl ether). In the study reported below, we have repeated the work of Simon *et al.* (4) and have examined some of the properties of the opiate-binding material. Our results indicate that a large number of distinct membrane components are capable of stereospecifically binding opiates *in vitro*.

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MATERIALS AND METHODS

Preparation of brain tissue fractions. Lysed mitochondrial and microsomal fractions were isolated from mouse brain as previously described (5). Since both of these fractions are rich in stereospecific opiate binding (5, 6), they were used interchangeably in the experiments described below. The only significant difference observed between the two was a higher specific activity (stereospecific binding per mg of protein) and a higher percentage of displacement by excess unlabeled opiate (stereospecific binding/total binding) in the microsomal fraction.

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Solubilization of opiate-binding material. This was carried out essentially as described by Simon *et al.* (4). Brain membranes, 1 mg protein/ml in 50 mM Tris,[1] pH 7.4, were incubated for 30 minutes at room temperature with 1-2 nM ^{3}H-enkephalinamide, or other radioactive ligand, and generally in the presence or absence of 100 nM levorphanol. Following incubation, the samples were centrifuged 15 minutes at 50,000 × *g* in a Sorvall RC2-B centrifuge, or 3 minutes at 15,000 × *g* (in a very small volume) in a Brinkmann Zentrifuge, and the pellet resuspended in ice-cold 50 mM Tris, pH 7.4-1% Brij 36-T, at a protein concentration of 5 mg/ml. After a 5 minute incubation at 0°, the suspension was centrifuged as before; under these conditions, levorphanol-displaceable radioactivity was found to be almost quantitatively extracted from the pellet, along with about 40% of the protein.

Further details or variations of this standard procedure are given in the RESULTS. We used levorphanol instead of the corresponding unlabeled peptide to displace ^{3}H-enkephalinamide (or ^{3}H-β_H-endorphin) binding, because then comparison could be made with the inactive isomer dextrorphan. However, in agreement with the results of others (7, 8), we found that although high concentrations of the latter had significantly less ability to displace these radioactive ligands than did corresponding concentrations of levorphanol (Fig. 4, below), there was no concentration of unlabeled drug such that levorphanol gave maximum displacement while dextrorphan gave none. Thus we defined stereospecific, or stereoselective, binding as the difference in cpm bound in the presence or absence of 100 nM levorphanol, which gave virtually complete displacement. While this value is somewhat higher than that obtained by the usual procedure of taking the difference in cpm bound in the presence of dextrorphan and the presence of levorphanol, we feel it is a more accurate indicator of stereospecific binding, since displacement observed with 100 nM dextrorphan should not be non-specific binding. Stereospecific binding calculated either way gave qualitatively similar results, as did that calculated by using excess unlabeled peptide to displace.

Gel filtration. Gel filtration of Brij-extracted binding material was carried out on columns of Sepharose 6B, Sephadex G-75 and Sephadex G-25. All columns were 1.5 × approximately 27 cm, and were pre-equilibrated and eluted with 50 mM Tris, pH 7.4-0.1% Brij 36-T, at 4°. The Sepharose 6B and Sephadex G-75 columns were calibrated by eluting from them under the same conditions a mixture of proteins of known molecular weights. Recovery of radioactivity from the columns was 85–95%.

Our studies, as well as those of Simon *et al.* (4) suggest that at low temperatures Brij-extracted opiate-binding material is stable over a period of many hours. In gel filtration experiments, however, in which Brij extracts of displaced and non-displaced binding were run in succession on the same column, the displaced sample was always eluted first. Since this sample has the lower amount of radioactivity, any dissociation of originally-bound radioactivity occurring over the period in which the experiment was carried out would lower, not raise, the values of stereospecifically-bound radioactivity subsequently calculated.

Analytical iso-electric focusing. Brij-extracted binding material was analyzed on iso-electric focusing gels in a pH gradient of 3-10 or 7-9 at 4°. Gels (5 × 80 mm) were cast from a solution containing 5% acrylamide, 0.13% bisacrylamide, 1.0% Brij, 36-T, 2% ampholine, 0.02% ammonium persulfate, and 0.02% N,N,N′,N′-tetramethylethylenediamine (TEMED). Samples were applied to the top of the gel, overlaid with cathodal buffer (1.6% mono-ethanolamine), and run for 3 hours at 100 V and 1-2 hours at 200 V between the mono-ethanolamine and 0.2% H_2SO_4.

At the end of electrophoresis, the gels were sliced into 2 mm segments, and each segment allowed to incubate overnight in 0.5 ml of 0.1% Brij 36-T. Following incubation, the pH of each eluant was determined with a Beckman pH meter, and then the sample was assayed for radioactivity as pre-

[1] The abbreviations used are: Tris, tri-hydroxy amino methane; TEA, tri-ethanolamine; SDS, sodium dodecyl sulfate; DMS, dimethyl suberimidate; TEMED, N,N,N′,N′-tetramethylethylenediamine.

viously described (5). Recovery of radioactivity from gels was 90–100%.

Under these conditions of electrophoresis, some deviation from perfect linearity was observed in the pH gradient, and the gradient in the 3–10 system did not extend above 9.0 (see RESULTS). This did not appear to be the result of a failure to reach equilibrium, because essentially the same pH gradients and the same radioactivity profiles were observed when duplicate samples were run for different lengths of time, including the appearance of a component at the very bottom of the gel. The most likely explanation is that the gradients were affected by the relatively large amounts of near-neutral pH solutions that had to be loaded onto these gels.

Preparative iso-electric focusing. This was carried out in a discontinuous sucrose gradient formed within a nominally 110 ml capacity ISCO column, equipped with inner and outer circulating water jackets. The column was filled, from bottom to top, with the following solutions: 30 ml 0.02% H_2SO_4-41% sucrose; 10 ml 37% sucrose; 10 ml 33% sucrose; 10 ml 29% sucrose; 10 ml 25% sucrose; 10 ml 21% sucrose; 10 ml 17% sucrose; 10 ml 13% sucrose; 10 ml 9% sucrose; and 20 ml 1.6% mono-ethanolamine-5% sucrose. The 37%, 33%, 29% 21%, 17%, 13%, and 9% sucrose solutions also contained 2% ampholine (pH 3–10) and 0.1% Brij 36-T, while the 25% sucrose solution contained the solubilized binding material, in 50 mM Tris, pH 7.4-1.0% Brij 36-T.

The column was maintained at 4°, and run at 250 V for about 12 hours, and 400 V for 16–18 hours. At the end of electrophoresis, 2.5 ml fractions were collected from the bottom, and these were analyzed for pH, absorbance at 280 μ, and radioactivity.

Cross-linking of radioactive peptides to solubilized membrane components. Membranes were incubated with 1 nM ^{3}H-enkephalinamide or ^{3}H-β_H-endorphin in the presence or absence of 100 nM levorphanol; following centrifugation, extraction of the pellet with 1% Brij-50 mM tri-ethanolamine (TEA) pH 8.5 at 0°, and re-centrifugation, the solubilized material was incubated with 10 mM dimethyl suberimidate (DMS) for 2 hours at 0°. The samples were then made 1% in SDS, 1% in β-mercaptoethanol, boiled, and analyzed by SDS gel electrophoresis as previously described (9).

Assays. Protein and radioactivity were assayed as previously described (9).

Materials. ^{3}H-methionine-enkephalinamide (30–50 C/mmole), ^{3}H-etorphine (20 C/mmole) and ^{3}H-naloxone (11 C/mmole) were from New England Nuclear, Boston, Mass. ^{3}H-β_H-endorphin (50–100 C/mmole) was prepared from synthetic iodinated analogues by catalytic exchange (10). Brij 36-T was from Emulsion Engineering, Elk Grove, Ill. or from Sigma, St. Louis, Mo. (the latter company no longer supplies this chemical). Gel filtration resins were from Pharmacia, Piscataway, N. J. Ampholine solutions were from LKB Produkter, Pleasant Hill, Ca. Mono-ethanolamine was from Baker, Oakland, Ca. Sources of other materials have been given previously (5, 9).

RESULTS

Gel filtration of solubilized opiate binding components. Brain membranes were incubated with 1 nM ^{3}H-enkephalinamide in the presence or absence of 100 nM levorphanol, then extracted with Brij 36-T, as described in MATERIALS AND METHODS. When the solubilized material was applied to a column of Sepharose 6B, a peak of levorphanol-displaceable radioactivity was observed in the molecular weight range 100,000–500,000 (Fig. 1a). The size of this peak, and the fraction of total radioactivity that it contains (approximately 25%) are both in good agreement with the results of Simon *et al.* (4); however, the very broad distribution of radioactivity in Fig. 1a suggested heterogeneity, and because Sepharose 6B does not fractionate proteins of less than 5,000–10,000 molecular weight, it is not clear from Fig. 1a whether the large peak contained all of the bound radioactivity applied to the column.

Filtration on Sephadex G-75 resolved the levorphanol-displaceable radioactivity into several components (Fig. 1b); a major peak was excluded, which presumably corresponded to the high molecular weight material observed on Sepharose 6B, but several partially-included peaks were also observed, in particular two of about 6,000 and

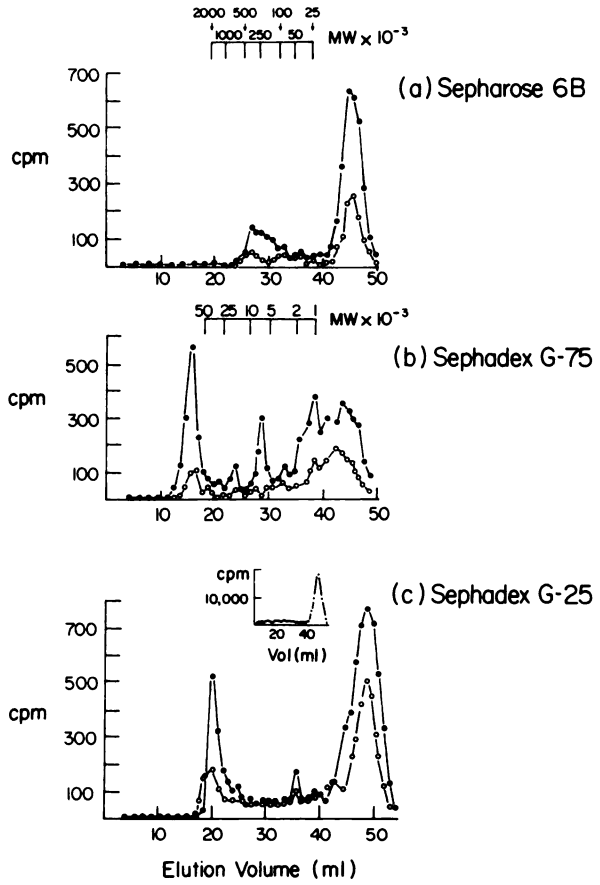

FIG. 1. *Gel filtration of Brij-extracted opiate binding material*

Brain membranes were incubated with 2 nM ^{3}H-enkephalinamide, then extracted with 1% Brij 36-T, as described in MATERIALS AND METHODS. One milliliter of the solubilized material (about 2 mg protein) was applied to the indicated column, and eluted at 15–20 ml/hr with 50 mM Tris-0.1% Brij 36-T, at 4°. Fractions (1.0 ml) were collected and aliquots assayed for radioactivity. ●——●, original incubation in the absence of 100 nM levorphanol; ○——○, original incubation in the presence of levorphanol. (c), Inset is the profile of ^{3}H-enkephalinamide eluted alone on G-25. The Sepharose 6B and Sephadex G-25 columns were calibrated by eluting from them, under the same conditions as given above, a mixture of the following standard proteins: Phosphorylase a, hexokinase, hemoglobin, ovalbumin and chymotrypsinogen (Sepharose 6B); hemoglobin, ovalbumin, chymotrypsinogen and RNAse II (Sephadex G-75).

1,500 molecular weight, respectively. In this experiment, 75% of the levorphanol-displaceable radioactivity was recovered in a bound form (although, as in Fig. 1a, only about 25% of the total radioactivity was bound). Virtually all of the levorphanol-displaceable radioactivity was excluded from Sephadex G-25, which nominally voids globular species of greater than 1500 molecular weight, but some non-displaceable radioactivity was included in the column (Fig. 1c). In all of these systems, unbound ^{3}H-enkephalinamide was completely retained by the column (Fig. 1c, inset).

Iso-electric focusing of solubilized opiate-binding material. When 1% Brij extracts of brain membranes incubated with ^{3}H-enkephalinamide in the presence or absence of levorphanol were analyzed by isoelectric focusing, a broad spectrum of displaceable radioactivity was observed (Fig. 2). On 5% gels in the pH 3–10 system, major peaks of pI 8.6, 8.4 and 3.2 were observed, as well as a diffuse area in the pI range 8.3–

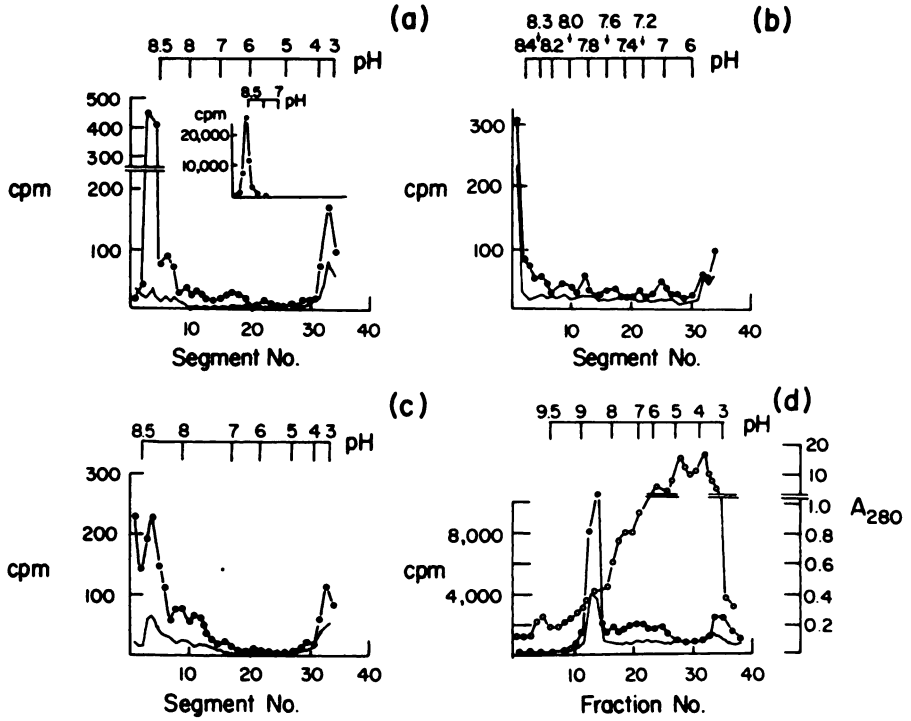

FIG. 2. *Isoelectric focusing of Brij-extracted opiate binding material*

Brain membranes were treated exactly as given in the legend to Fig. 1. (a) Fifty microliters of the solubilized material (about 100 μg protein) applied to gels and run in the pH 3–10 system, as described in MATERIALS AND METHODS; (b), as in (a), except the pH 7–9 system was used; (c), 1.0 ml of solubilized material passed down a Sephadex G-25 column, as in the legend to Fig. 1, then 200 μl of bound eluate (about 100 μg protein) run as in (a); (d), 10 ml of the solubilized material run in the preparative pH 3–10 system, as described in MATERIALS AND METHODS. (a), inset, ^{3}H-enkephalinamide alone applied to analytical pH 3–10 system. Preparation and analysis of fractions is described in MATERIALS AND METHODS. In (a)–(d), sample originally incubated in absence (●——●) or presence (——) of 100 nM levorphanol. In (d), O——O, A_{280}.

6.5 (Fig. 2a); the very large peak at pI 8.6 was due at least in part to free ^{3}H-enkephalinamide (cf. with inset, Fig. 2a), while the other peaks must represent bound material. A basically similar profile was observed in the pH 7–9 system which, however, also failed to fractionate effectively the diffuse area in the pI range 8.3–6.5 (Fig. 2b).

The radioactivity in the gel profiles of Fig. 2a and 2b is very low, but these patterns were reproducible in 6–8 experiments. Furthermore, a similar profile was observed when the gels were loaded with the excluded material from a G-25 column (Fig. 1c), which removes free ligand. In this profile (Fig. 2c) major peaks at pI's of 8.4 and 3.0 are very clear, and minor peaks of pI's about 8.0, 7.7 and 7.0 can also be observed, most of which appear to be present also in the profiles of Fig. 2a and 2b. Some material also appears at a pI greater than 8.4, although this could be free ligand resulting from slight dissociation. Finally, a somewhat similar profile was also observed following preparative iso-electric focusing in the pH 3–10 system (Fig. 2d) or 7–9 system (not shown). Although the major pI 8.4 peak was not resolved from ^{3}H-enkephalinamide under these conditions, its presence was demonstrated by the appearance of high molecular weight material in this peak (Fig. 3b, below).

Relationship of binding components identified by gel filtration and by iso-electric focusing. When the three major bind-

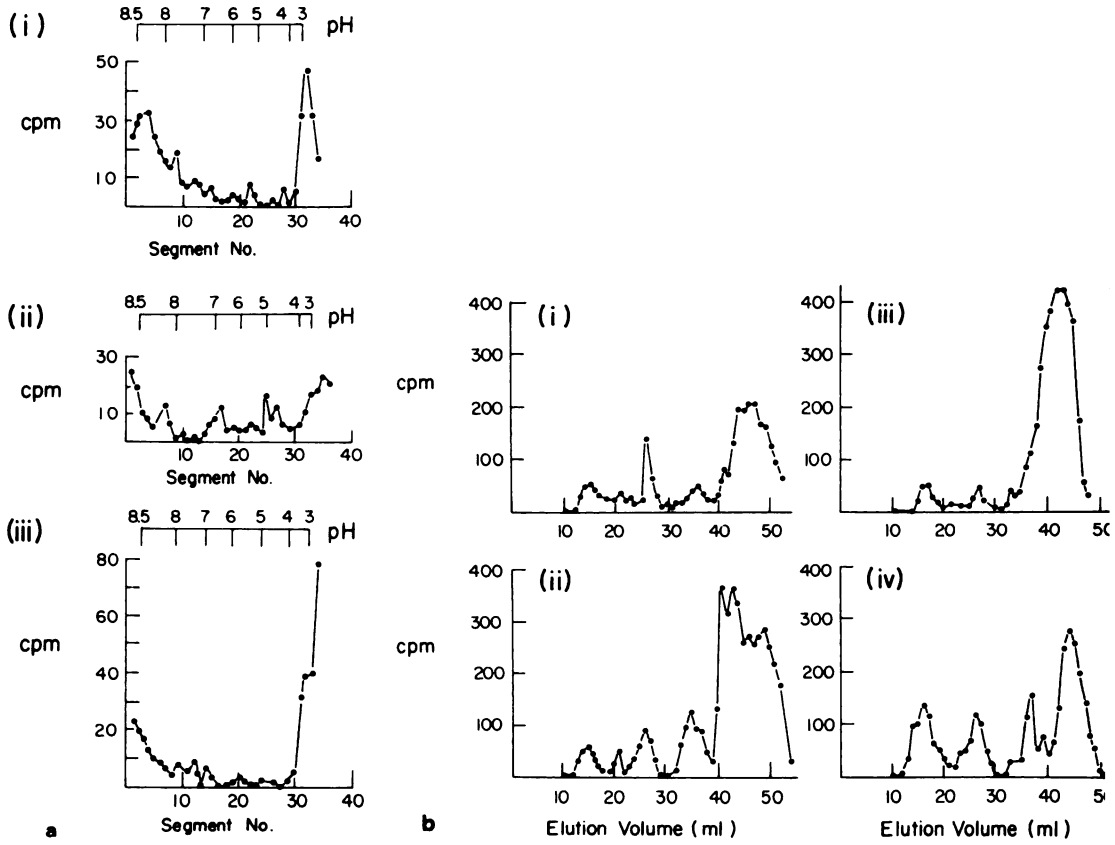

FIG. 3. *Analysis of Brij-extracted opiate binding material by both gel filtration and isoelectric focusing*
Brain membranes were incubated and extracted exactly as given in the legend to Fig. 1. (a), 1.0 ml of solubilized material was passed down a Sephadex G-75 column, as in the legend to Fig. 1b; 100 μl aliquots of various fractions were run on pH 3–10 isoelectric focusing gels, as described in MATERIALS AND METHODS. (i), Fraction eluting at 16 ml; (ii), fraction eluting at 29 ml; (iii), fraction eluting at 39 ml. (b), 10 ml of solubilized material applied to pH 3–10 preparative isoelectric focusing system as described in MATERIALS AND METHODS, and 1.0 ml aliquots of various fractions passed down a G-75 column, as in the legend to Fig. 1b. (i), fractions pI 8.8–8.4 (pooled); (ii), fraction pI 8.1; (iii), fraction pI 7.0; (iv), fractions pI 3.6–3.0 (pooled). Original incubations were carried out in the absence and presence of levorphanol, but only the former samples were subsequently analyzed. Displacement of total binding to membranes was about 75%.

ing components observed on G-75 Sephadex (Fig. 1b) were analyzed by iso-electric focusing, each was observed to be heterogeneous (Fig. 3a); in fact, in each case the profile was comparable to that obtained using total binding material (Fig. 2a, c), containing both major pI peaks as well as minor species. In this experiment, only samples incubated in the absence of levorphanol were analyzed, but the displacement by levorphanol of total binding to membranes before extraction was about 75%.

In the case of the highest molecular weight material, some of this heterogeneity might have arisen from association of lower molecular weight components, such as lipids, with proteins, under the conditions of gel filtration; thus the pI component which predominates in the lowest molecular weight G-75 fraction (Fig. 3a (iii)) might be present in higher molecular weight fractions only as a result of association. However, when an experiment of reverse design was carried out, in which fractions prepared by preparative iso-electric focusing were subsequently analyzed on G-75, it was

found that the low pI material, as well as that of higher pI, contained both high and low molecular weight components (Fig. 3b).

Sensitivity of solubilizable binding components to unlabeled opiates and to Na^+. Brain membranes were incubated with ^{3}H-enkephalinamide in the presence or absence of a number of different unlabeled opiates, each at 100 nM, then extracted with Brij 36-T and analyzed by iso-electric focusing (Fig. 4). The pI 8.4 component was not well resolved from unbound ligand in these experiments, but binding to it and certainly to the other species was significantly displaced by each labeled ligand, although the amount of displacement occurring among the different components was somewhat variable (Fig. 4b–e). Because of the low level of cpm on these gels, we used concentrations of these drugs that would be expected to achieve virtually maximal displacement in each case; thus it was not possible to compare their affinities for the various binding components, although in general they appeared to follow the order levallorphan~methadone>oxymorphone>pentazocine, the same order observed with total membrane binding. The inactive isomer dextrorphan was much less potent in displacing binding from each component (Fig. 4a), while Na^+ at a concentration of 100 mM drastically reduced levorphanol-displaceable binding to each component (Fig. 4f).

Binding components sensitive to other labeled opiates. Brain membranes were incubated with 1 nM ^{3}H-β_H-endorphin, ^{3}H-etorphine, or ^{3}H-naloxone in the presence or absence of 100 nM levorphanol, extracted with 1% Brij 36-T, and analyzed by iso-electric focusing (Fig. 5). In each case, displaceable ligand was found associated with a wide range of components, somewhat similar to that binding ^{3}H-enkephalinamide (Fig. 2a, 2c). Thus all three ligands bound to major components of pI's 8.4–8.6 and about 3, as well as minor components in the pI range 8.5–6.0. It is not clear from our results whether the binding components to each of the four ligands tested are the same, but clearly they are very similar in this system.

Covalent labelling of stereospecific opiate binding components. Brian membranes

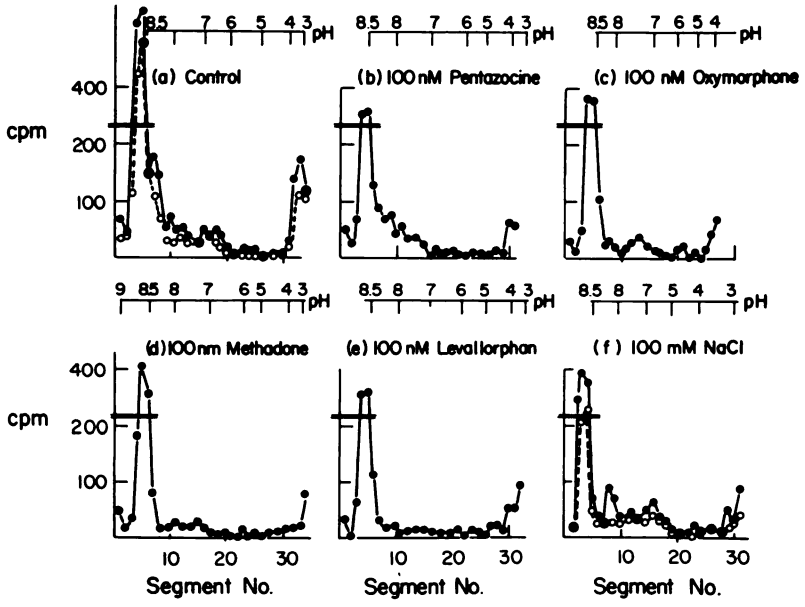

FIG. 4. *Effect of unlabeled opiates and Na^+ on ^{3}H-enkephalinamide binding components*

Analysis was exactly as given in the legend to Fig. 2a, except that original incubation of membranes with 1 nM ^{3}H-enkephalinamide was in the presence of the following: (a), no additions (●——●) or 100 nM dextrorphan (○——○); (b)–(e) 100 nM of the indicated unlabeled drug; (f), 100 mM NaCl in the absence (●——●) or presence (○——○) of 100 nM levorphanol.

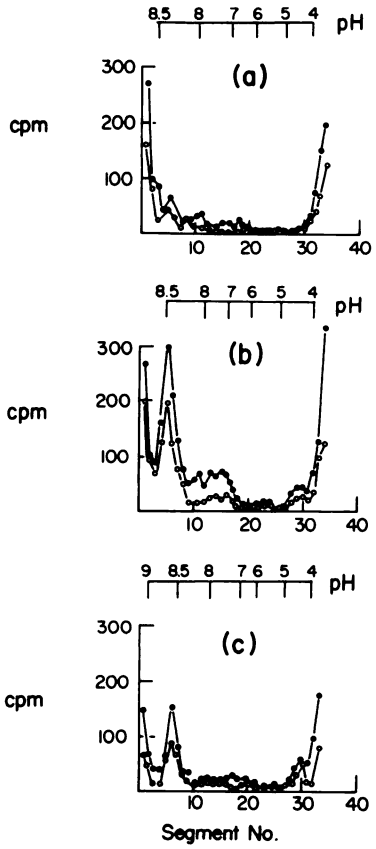

FIG. 5. *Binding components sensitive to labeled opiates*

Analysis exactly as given in the legend to Fig. 2a, except that the original incubation of brain membranes was with (a), 1 nM ^{3}H-B_H-endorphin; (b), 1 nM ^{3}H-etorphine; (c), 1 nM ^{3}H-naloxone. Original incubations were in the absence (●——●) or presence (○——○) of 100 nM levorphanol.

were incubated with ^{3}H-β_H-endorphin, solubilized in Brij, then cross-linked with DMS, as described in MATERIALS AND METHODS. Analysis of the cross-linked material on SDS polyacrylamide gels revealed a broad range of displaceable-binding, covalently-labeled components (Fig. 6); no covalent labeling was observed if DMS were added to the Brij extract immediately prior to solubilization with SDS and boiling (Fig. 6a). Electrophoresis on 7.5% gels indicated the presence of binding components of up to more than 150,000 molecular weight (Fig. 6a), while analysis of the material on 12% gels demonstrated components of very low molecular weight (Fig. 6b). Considerably less cross-linking was observed with the shorter reagent dimethyl adipimidate.[2]

DISCUSSION

Extraction of brain membranes with 1% Brij 36-T removes up to 75% of the stereospecifically-bound ^{3}H-enkephalinamide (Fig. 1). The binding material is clearly heterogeneous with respect to both size and charge (Figs. 1–3), yet all of the identifiable components show sensitivity to a number of different opiates, as well as to Na^+ (Fig. 4). Heterogeneity is also observed when ^{3}H-β_H-endorphin, ^{3}H-etorphine, or ^{3}H-naloxone is used (Figs. 5, 6).

While it is possible that some of this heterogeneity might have arisen as a result of association of a few distinct small molecules into complexes of different size and pH, this cannot be a complete explanation of our data, because even the very lowest molecular weight components show heterogeneity with respect to charge (Fig. 3a (iii)) and, furthermore, heterogeneity is observed both in analytical and preparative focusing systems (Fig. 2), which were run with different detergent concentrations. Association might account for the presence of some or all of the high molecular weight components, but we feel that this is unlikely because of the protein-like nature of the material (4) and because such high molecular weight material is observed on columns run in both the presence and absence of detergent (Fig. 1) (4). We conclude that stereospecific opiate binding, as measured *in vitro,* comprises a number of distinct components, probably including both lipids and proteins.

Both Simon *et al.* (4) and Zukin and Kream (11) claimed that a single high molecular weight binding component of 370,000–380,000 molecular weight was observed following Brij extraction, but neither group actually published its Sepharose 6B profiles. Zukin and Kream (11), furthermore, observed a major 35,000 molecular weight species following cross-linking with DMS of ^{125}I-enkephalinamide to Brij-extracted material purified on Sephadex columns; however; this purified material con-

[2] Smith, A. P., unpublished data.

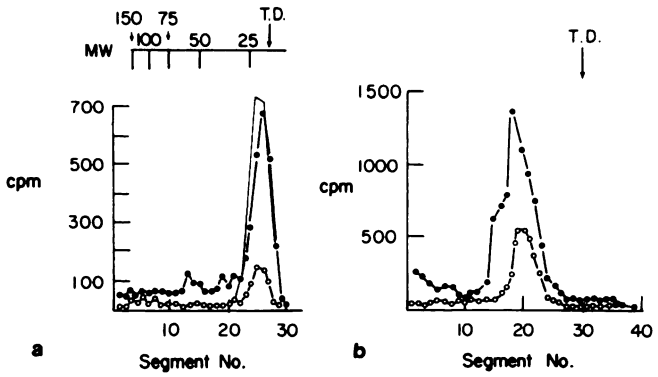

FIG. 6. *Cross-linking of ^{3}H-B_H-endorphin to opiate binding components*

Brain membranes were incubated with 1 nM ^{3}H-B_H-endorphin in the presence or absence of 100 nM levorphanol, then extracted with 1% Brij and incubated with 10 mM DMS, as described. Samples were then analyzed by SDS gel electrophoresis as in (9). (a), 7.5% gels; (b), 12% gels. In (a) and (b), (●——●) absence and (○——○) presence of levorphanol, and in (a), (——) DMA added immediately prior to electrophoresis. T.D., tracking dye position. About 50 μg protein loaded on gel (a), 100 μg on (b). The gel in (a) was calibrated for molecular weight as in (9); the gel in (b) was not calibrated but excludes material of greater than approximately 15,000 molecular weight. The major peak in both systems is free ^{3}H-B_H-endorphin.

tained only 10% of the cpm originally bound to the Brij-extracted material, so clearly a specific component could have been selected for. It should be pointed out that enkephalinamide is a poor choice for this type of experiment, since after reaction with suberimidate it no longer possesses the free amino group that is presumably necessary for binding. We have repeatedly been unable to cross-link either this ligand or β-endorphin to intact membranes,[2] which supports the original contention of Simon *et al.* (4) that the ligand receptor complex is altered in some significant manner after extraction from the membrane with Brij.

Opioid ligands are known to react with several classes of binding sites, differing in affinity and capacity (12, 13) or in specificity (7, 8, 14–16), and our data may in part reflect the existence of such receptors. However, since our experiments were carried out at radioactive ligand concentrations that would greatly favor high affinity binding, and since heterogeneous and quite similar binding profiles were observed with alkaloid agonists and antagonists, as well as peptides, we believe the heterogeneity described by our results is of a much greater order than that which can be accounted for by a few classes of receptors differing in some binding property. It seems unlikely that all of these components would be relevant to pharmacological activity *in vivo*, but our results strongly suggest that the initial interaction of opiates with nerve cells is a highly complex process.

ACKNOWLEDGMENT

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