Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 4138–4142, June 1986
Biochemistry

Purification to apparent homogeneity of a μ-type opioid receptor from rat brain

(affinity chromatography)

TAE MOOK CHO, JUN-ICHI HASEGAWA, BANG-LUN GE, AND HORACE H. LOH

Departments of Psychiatry and Pharmacology and Langley Porter Neuropsychiatric Institute, University of California, San Francisco, CA 94143

Communicated by John J. Burns, February 5, 1986

ABSTRACT **A μ-opioid-specific receptor was purified to apparent homogeneity from rat brain membranes by 6-succinylmorphine affinity chromatography, gel filtration, wheat germ agglutinin affinity chromatography, and isoelectric focusing. The purified receptor had a molecular weight of 58,000 as determined by NaDodSO$_4$/polyacrylamide gel electrophoresis and was judged to be homogeneous by the following criteria: (*i*) a single band was detected by autoradiography after NaDodSO$_4$/polyacrylamide gel electrophoresis of ^{125}I-labeled receptor and (*ii*) the purified preparation had a specific opioid-binding activity of 17,720 pmol/mg of protein, close to the theoretical value. In addition, the M_r 58,000 value agrees closely with that determined by covalently labeling purified receptor with bromoacetyl-[^{3}H]dihydromorphine or with ^{125}I-labeled β-endorphin and dimethyl suberimidate.**

Although the opiate receptor was identified more than 10 years ago (1–3), its purification has proved to be an extremely difficult task. For one thing, the receptor is inactivated by many of the relatively gentle detergents that have been successfully used to solubilize other receptors (4–6). Adding to the difficulty is receptor heterogeneity. At least three different receptor types exist in mammalian brain (7–9), and their relationships to one another are unclear. That is, it has not been established whether one or more of these types coexist on a single macromolecule (10) or whether each receptor type is located on a distinct molecule (11).

Recently, considerable progress in opiate receptor purification has been made in several laboratories, but complete purification of an "active" receptor—capable of binding opiates *in vitro*—has, to our knowledge, not yet been achieved. Thus, one group has reported complete purification of the δ-type (enkephalin-selective) opiate receptor from neuroblastoma–glioma hybrid cells, but this is an inactive preparation containing a covalently bound affinity ligand (12) and therefore cannot be used to study opiate receptor function. Conversely, isolation of active receptor has been reported by several other laboratories, but in each case the binding properties have not been characterized extensively and the binding activity of the preparation indicates that the receptor has not been purified to theoretical homogeneity (13–17). In addition, the kinetic binding parameters of these isolated preparations, such as K_d, generally differ significantly from those of the membrane-bound opiate receptors.

Several years ago, our laboratory reported an effective method of receptor solubilization, involving sonication as well as detergent (17). Using affinity chromatography on Affi-Gel 102 coupled to 6-succinylmorphine, we were subsequently able to partially purify opiate-binding material from this extract and demonstrate that it exhibited many properties similar to those of membrane-bound opiate receptors. Thus, the binding to the isolated receptor was stereospecific, of high affinity, and sensitive to proteolytic agents; most important, the rank order of affinities of a series of opiate ligands was similar for the two preparations (16, 17). These data strongly suggested that the purified preparation contained genuine opiate receptors. We also demonstrated that the isolated opiate-binding material contained lipids as well as proteins, and these were apparently critical to its activity. This is consistent with a large and diverse body of evidence indicating that both components are necessary for binding to membrane-bound opiate receptors (18–20). We now report purification of this material to apparent homogeneity. It has a molecular weight of 58,000 and shows binding properties similar to those of the membrane opioid receptor of the μ type.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Materials. [^{3}H]Diprenorphine (43 Ci/mmol; 1 Ci = 37 GBq), [[^{125}I]iodotyrosine-27]β-endorphin (human, 2000 Ci/mmol), and Na^{125}I (13.3 mCi/μg of iodine) were from Amersham. [*N-methyl*-^{3}H]Dihydromorphine (86 Ci/mmol) and 1-fluoro-2,4-dinitro[3,5-^{3}H]benzene (20.4 Ci/mmol) were from New England Nuclear. *N*-acetylglucosamine, acrylamide, *N,N'*-methylenebis(acrylamide), bovine serum albumin, phosphatidylserine, phosphatidylinositol, and phosphatidylinositol 4,5-bisphosphate were from Sigma. Affi-Gel 102 was from Bio-Rad; Ultrogel AcA 34 was from LKB; sodium dodecyl sulfate was from Baker; and agarose-bound wheat germ agglutinin was from Vector Laboratories (Burlingame, CA). Morphine was obtained from Mallinckrodt. Cerebroside sulfate was isolated from bovine brain as described (21). The 6-succinylmorphine derivative of Affi-Gel 102 was prepared as described (17). 6-Bromoacetyl-[^{3}H]dihydromorphine was synthesized by stirring 1 mCi of [^{3}H]dihydromorphine (86 Ci/mmol) and 0.1 ml of bromoacetyl bromide in 0.5 ml of acetonitrile under nitrogen overnight; it was purified by thin-layer chromatography using silica gel and acetonitrile.

Affinity Chromatography on 6-Succinylmorphine-Affi-Gel 102 (Step I). Isolation of the partially purified opiate receptor protein was carried out as previously described (17). P_2 fractions prepared from eight rat brains minus cerebella were suspended in 180 ml of ice-cold 0.32 M sucrose. The suspension was sonicated, and then Triton X-100 was added to a final concentration of 0.5%. After incubation at 0°C for 30 min, the sample was centrifuged at 100,000 × *g* for 1 hr, and the supernatant was loaded onto the Affi-Gel column equilibrated at room temperature. The column was washed with 1.5 liters of 50 mM Tris·HCl (pH 7.4), followed by elution with a 500-ml linear gradient of NaCl [0–1 *M* in 50 mM Tris·HCl (pH 7.4)]. Fractions were assayed for ^{3}H-labeled-opiate binding activity (see below), and those with appreciable activity were pooled, concentrated, and used for further purification.

Gel Filtration (Step II). An Ultrogel AcA 34 column (M_r range 20,000–350,000) was prepared and washed extensively with 2 liters of 50 mM Tris·HCl (pH 7.4). Five milliliters (≈20 mg of protein) of the concentrated opiate-binding fraction isolated from the Affi-Gel column was loaded onto this AcA column and eluted with the Tris buffer. Fractions (4.4 ml) were collected and assayed for protein and for opiate binding.

Affinity Chromatography on Wheat Germ Agglutinin-Agarose (Step III). Ten milliliters of agarose-bound wheat germ agglutinin was packed in a 1.0 × 15 cm column and washed with 150 ml of Tris buffer. Three milliliters of the concentrated opiate-binding material (fractions 38–45) eluted from the AcA 34 column were applied to the wheat germ column, and elution with the Tris buffer was continued until a peak of unbound protein was removed. The latter was reapplied to the column, and elution was continued as before. After repeating this procedure 10 times, bound material was removed by elution with 1 M *N*-acetylglucosamine. Fractions with opiate-binding activity were pooled and concentrated by Amicon filtration.

Isoelectric Focusing (Step IV). Isoelectric focusing of the isolated material was carried out by the procedure of Maizel (22). The sample, also containing 1% (wt/vol) Ampholines (pH 3–10, LKB) and 10% (vol/vol) glycerol, was layered on top of a 4% polyacrylamide gel and electrophoresed for 30 min at 200 V and 20 hr at 400 V, between 0.2% sulfuric acid and 0.4% ethanolamine. After electrophoresis, the gel was sliced into segments, the pH values of which were determined with a surface electrode. Then proteins were eluted from each segment with 1 ml of 10 mM sodium phosphate, pH 8.0/100 mM NaCl, and opiate binding activity of each eluate was determined.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. In order to determine the purity of the opiate receptors, the isolated material was iodinated with Na^{125}I (2000 Ci/mmol) in the presence of chloramine-T (23) and then separated from free ^{125}I with a Sephadex G-15 column (0.5 × 20 cm). The iodinated material was analyzed by NaDodSO$_4$ polyacrylamide gel electrophoresis by the procedure of Laemmli (24), using 10% separating gel and 4% stacking gel. The gel was fixed and dried under vacuum and exposed to x-ray film (Kodak X-Omat AR, 5 × 10 inches) for 20 days at −70°C.

Affinity Labeling. Samples of the purified opiate receptor were incubated with 10 nM 6-bromoacetyl-[^{3}H]dihydromorphine (86 Ci/mmol) in 50 mM sodium phosphate (pH 8.0) for 1 hr at 37°C in the presence or absence of acidic lipids (40 μg). The incubation mixtures were then analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography, as described above. Alternatively, crosslinking experiments were performed by incubating the samples with ^{125}I-labeled β-endorphin (2 nM) and dimethyl suberimidate (1 mM) in 50 mM sodium phosphate (pH 8.0) for 30 min at room temperature; samples then were processed as above.

Opiate-Binding Assays. Binding experiments were carried out as previously described (17). In brief, samples contained the material to be assayed, 40 μg of acidic lipids [cerebroside sulfate/phosphatidylinositol 4,5-bisphosphate/phosphatidylinositol/phosphatidylserine, 2:1.5:1:1 (wt/wt)], and 2 nM [^{3}H]diprenorphine in 50 mM Tris·HCl (pH 7.4). Samples were incubated, in the presence or absence of excess unlabeled diprenorphine, for 30 min at 25°C and 20 min at 0°C. Then 0.2 ml of Tris buffer containing 2.6% charcoal and 2% bovine serum albumin was added, and the samples were mixed on a Vortex for 5 sec and centrifuged for 3.5 min in a Brinkmann desk-top centrifuge. An aliquot (0.5 ml) of each supernatant was added to 9 ml of scintillation fluid (Scintiverse II, Fisher Scientific, Santa Clara, CA), and radioactivity was determined in a liquid scintillation counter. Specific [^{3}H]diprenorphine binding was defined as the difference between binding (cpm) in the absence and in the presence of 10 μM unlabeled diprenorphine.

Protein Assay. Protein in the brain membranes and in step I-purified material was determined by the procedure of Lowry *et al.* (25), using bovine serum albumin as standard. The protein isolated in the later purification stages was labeled with 1-fluoro-2,4-dinitro[3,5-^{3}H]benzene (26) and purified by gel filtration on Sepharose 2B (0.5 × 25 cm). The radioactivity of this material was used to estimate the amount of protein present.

RESULTS

Affi-Gel Affinity Chromatography (Step I) and Gel Filtration (Step II). As previously reported (17), chromatography of the solubilized brain membranes on Affi-Gel 102 with a linear gradient of NaCl yields two protein peaks. Peak A was eluted at 0.35 M NaCl (fractions 10–20), and peak B at 0.6 M NaCl (fractions 22–34). Moreover, the peak A proteins, but not the peak B material, exhibited significant opiate-binding activity. Consequently, the peak A fractions were pooled and concentrated by Amicon filtration for further purification. The pooled material, containing about 1.8% of the total protein in the original solubilized material, had a specific activity of 110 pmol of opiate bound per mg of protein, corresponding to a 420-fold purification over the original P_2 fraction. Assuming the molecular weight of the binding material to be 58,000 (see below), this corresponds to 0.6% of theoretical purity.

The peak A protein isolated by Affi-Gel affinity chromatography was further purified on Ultrogel AcA 34 (Fig. 1). Most of the proteins were eluted near the void volume, indicating a molecular weight higher than 350,000. However, most of the binding activity was eluted at a molecular weight of about 60,000 (fractions 38–45). After pooling and concentration, this material was found to bind 2200 pmol of opiate per mg of protein, corresponding to 8460-fold purification. Based on a molecular weight of 58,000, this indicates the material has attained about 13% of theoretical purity.

In addition to the opiate-binding material eluted at $M_r \approx$ 60,000 (peak II) another peak (peak I), at $M_r \approx$ 110,000, was also observed (fractions 32–37 in Fig. 1). When the peak I material was pooled, concentrated, and rechromatographed on the same column, the same two peaks of binding activity were observed (Fig. 2). This result, together with the observation that peak I corresponds to a molecular weight about

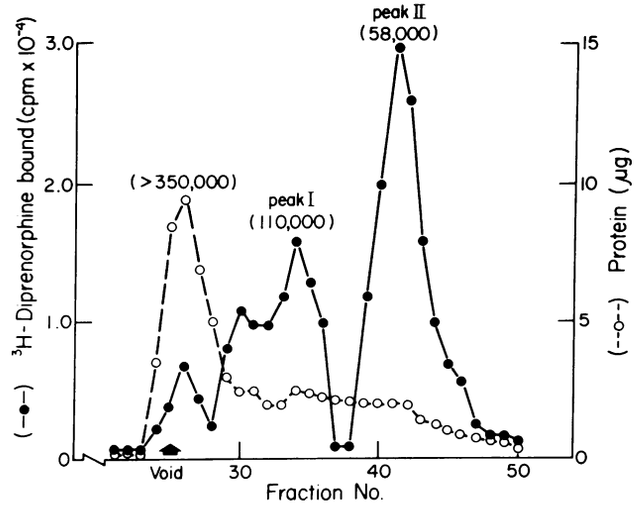

FIG. 1. Elution profile of the step I-purified receptor on Ultrogel AcA 34. Molecular weight values (in parentheses) were estimated from the elution volumes of the following standards: bovine serum albumin, transferrin, ovalbumin, and gamma globulin.

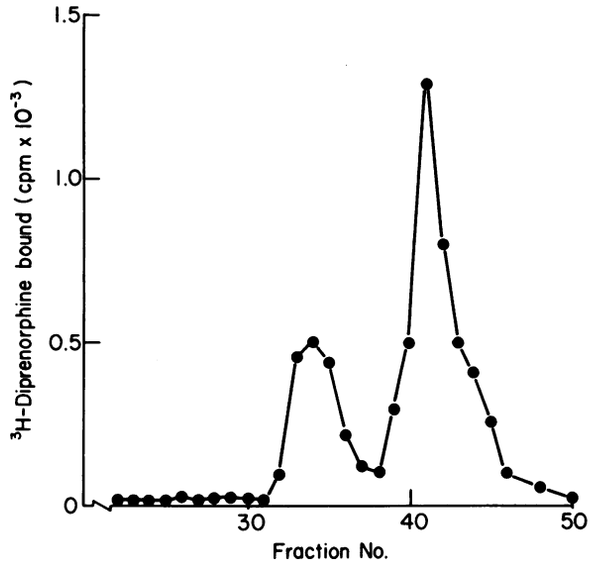

FIG. 2. Elution profile upon rechromatography of the M_r 110,000 fraction from step II.

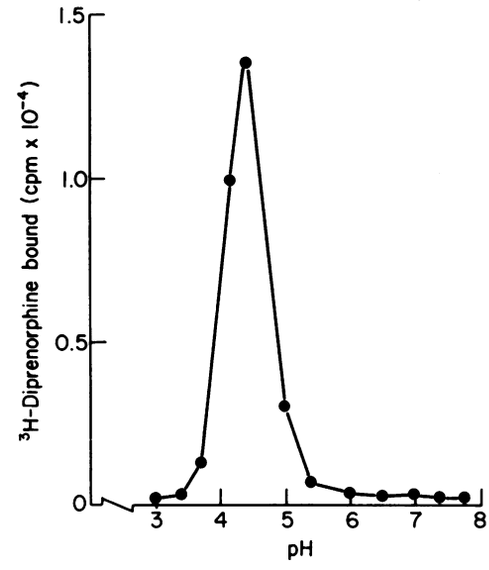

FIG. 4. [^{3}H]Diprenorphine binding profile of the opioid receptor after isoelectric focusing of the step III (wheat germ agglutinin)-purified material.

twice that for peak II, strongly suggests that the peak I material is an aggregate of the peak II material and not a distinct species.

Affinity Chromatography on Wheat Germ Agglutinin-Agarose (Step III). The peak II material obtained from gel filtration was subjected to further fractionation on a wheat germ agglutinin-agarose column (Fig. 3). [^{3}H]Diprenorphine binding was found in fractions 2–4. After pooling, concentration, and dialysis against Tris buffer, the material was found to have a specific activity of 9200 pmol per mg of protein. This corresponds to 35,380-fold purification, or 53% of theoretical purity.

Isoelectric Focusing (Step IV). The step III material was purified further by isoelectric focusing. As shown in Fig. 4, maximal opiate binding was associated with material focusing at pH 4.4, with a specific activity of 17,720 pmol per mg of protein. This is close to theoretical purity, assuming 1 mol of ligand binds to 1 mol of receptor of M_r 58,000 (see Table 1).

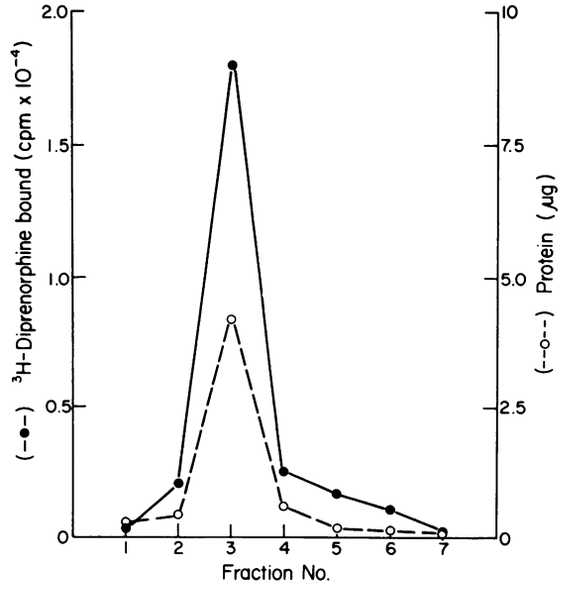

FIG. 3. Elution profile of the step II-purified opioid receptor on a wheat germ agglutinin-agarose affinity column. The M_r 60,000 material purified in step II was pooled, concentrated, and chromatographed on the wheat germ column.

Determination of Purity of Opiate-Binding Material. The binding activity per mg of protein of the material isolated in step IV is consistent with a completely purified protein but does not constitute direct proof of this conclusion. To verify that this material is, in fact, homogeneous, we iodinated the step IV material and analyzed it by NaDodSO$_4$/polyacrylamide gel electrophoresis. As shown in Fig. 5 (lane B), the radioactivity was associated with a single band, migrating at M_r 58,000. This is in good agreement with the data obtained by gel filtration in step II.

Association of Opiate-Binding Activity with the M_r 58,000 Protein. Although the above data indicate that the isolated material is nearly 100% pure, one could argue that the opiate-binding activity is actually associated with a minor species, not detectable by autoradiography after electrophoresis of the iodinated preparation. To establish that the M_r 58,000 protein observed on gels is the material responsible for binding opiates, step IV material was affinity-labeled with bromoacetyl-[^{3}H]dihydromorphine and analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis. The affinity-labeled material appeared in the same position as the iodinated protein, corresponding to M_r 58,000 (Fig. 5, lane C). This value also corresponds closely with that reported for the μ receptor by Newman and Barnard (27).

In another experiment, the receptor protein was cross-linked with iodinated β-endorphin by use of dimethyl

Table 1. Purification of μ opioid receptor

Step	Opiate binding, pmol/mg	Fold purification	% theoretical purity	Yield
P_2	0.26	1	0.001	100
I	110	423	0.6	70
II	2,200	8,460	13	39
III	9,200	35,380	53	17
IV	17,721	68,158	103	6

Protein and opiate binding were determined as given in *Materials and Methods*. For the P_2 fraction and step I and II material, the values are means of 3 determinations, and standard errors are 3% of the means. For step III and IV material, the values are means of 2 determinations, and standard errors are 30% of the means.

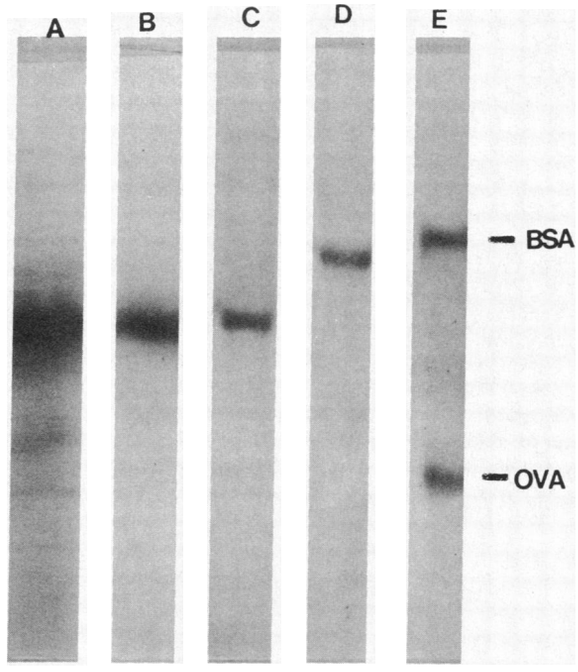

FIG. 5. Analysis of the purified opioid receptor by NaDodSO$_4$/polyacrylamide gel electrophoresis. Lanes: A, step III-purified receptor; B, step IV-purified receptor labeled with ^{125}I; C, step IV-purified receptor covalently labeled with bromoacetyl-[^{3}H]dihydromorphine; D, step IV-purified receptor covalently labeled with ^{125}I-labeled β-endorphin and dimethyl suberimidate; E, standard proteins used for molecular weight determinations [bovine serum albumin (BSA), M_r 66,000; ovalbumin (OVA), M_r 45,000].

suberimidate and analyzed by electrophoresis and autoradiography. The crosslinked material appeared at a position corresponding to M_r 63,000 (Fig. 5, lane D). When one takes into account the combined molecular weights of β-endorphin and suberimidate (about 4000), this value is close to the 58,000 found by the other procedures. This value is also in good agreement with that reported by Howard *et al.* (28), who applied a similar crosslinking procedure to the receptor present in unfractionated brain membranes.

DISCUSSION

In previous studies (16, 17, 29), opiate-binding material partially purified by affinity chromatography on 6-succinylmorphine coupled to Affi-Gel 102 had many properties similar to those of membrane-bound μ-type opioid receptors, including stereospecificity, high affinity, and relative affinities for a series of μ-opiate ligands paralleling those of membrane-bound receptors. Most important, its behavior on gel filtration columns showed that it was truly solubilized and not simply associated with small membrane vesicles or nonspecific aggregates. While recognizing that this material may comprise simply one subunit of a more complex molecule, we feel justified in referring to it as an "opiate receptor."

In the present study, we have taken this partially purified opiate receptor through four major fractionation steps, purifying it to apparent homogeneity. This conclusion is supported by (*i*) the appearance of a single band when the iodinated protein was analyzed by NaDodSO$_4$/polyacrylamide gel electrophoresis and autoradiography and (*ii*) a specific binding activity (pmol/mg of protein) close to that calculated for a M_r 58,000 protein binding opiate on a mole-per-mole basis. The latter criterion is admittedly not as strong as would be desired, because of a rather large error in protein determination of the purified receptor, which we found unavoidable when working with such small amounts of material. Further, as Simonds *et al.* (12) have pointed out, the presence of significant numbers of sugar residues in the receptor would result in an underestimation of the specific binding activity of theoretically pure material, if the calculation of specific activity is based on the molecular weight determined by hydrodynamic methods.

Further evidence that the M_r 58,000 species we have isolated is a genuine opiate receptor was provided by experiments in which an opioid ligand was covalently attached to the isolated protein, using either bromoacetyl-[^{3}H]dihydromorphine or ^{125}I-labeled β-endorphin and suberimidate. The apparent M_r of 58,000, moreover, agrees well with that reported for a μ-type opiate receptor identified by other groups (27, 28). It is of further interest that the affinity-labeled δ receptor recently purified by Simonds *et al.* (12) is also of M_r 58,000, although Howard *et al.* (28) claim that μ and δ receptors can be distinguished on the basis of molecular weight.

Although we have not completely characterized the purified opiate receptor, several characteristics of it became evident during the purification process: (*i*) the presence of acidic lipids, apparently noncovalently bound, which are essential for optimal opiate binding (17); (*ii*) the presence of glycosylated residues, which enable the protein to bind to wheat germ agglutinin (Fig. 2); and (*iii*) a pI of 4.4, suggesting a preponderance of acidic residues.

During the second step of purification, filtration on Ultrogel, we observed two peaks of opiate-binding activity, corresponding to molecular weights of about 58,000 and 110,000, respectively. Since refiltration of the M_r 110,000 material yielded the same two peaks, we conclude that the M_r 110,000 fraction is an aggregate containing the M_r 58,000 species. This aggregate could be a simple dimer of the M_r 58,000 protein or, alternatively, could consist of it and another species of similar molecular weight. The latter possibility is supported by our observation that the M_r 58,000 material, at step II of the purification, exhibits [^{3}H]GTP-binding activity (unpublished data). This suggests that it contains the GTP-binding regulatory subunit believed to be associated with the opiate receptor in neuroblastoma–glioma cells and in certain brain regions, which has a molecular weight of roughly 40,000 (30).

In conclusion, we have isolated a completely purified preparation of an opiate receptor that retains its ability to bind μ opiate ligands *in vitro*. With this material in hand, it will now be possible to characterize the receptor more extensively.

We thank Dr. Andrew Smith for critical comments, advice, and editing. This work was supported by National Institute on Drug Abuse Grant DA01583 and Research Scientist Award 5-KO5-DA70554 (H.H.L.).

1. Terenius, L. (1973) *Acta Pharmacol. Toxicol.* **32,** 317–320.
2. Pert, C. B. & Snyder, S. H. (1973) *Science* **179,** 1011–1014.
3. Simon, E. J., Hiller, J. M. & Edelman, I. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 1947–1949.
4. Ruegg, U. T., Hiller, J. M. & Simon, E. J. (1980) *Eur. J. Pharmacol.* **64,** 367–368.
5. Bidlack, J. M. & Abood, L. G. (1980) *Life Sci.* **27,** 331–340.
6. Simonds, W. F., Koski, G., Streaty, R. A., Hjelmeland, L. M. & Klee, W. A. (1980) *Proc. Natl. Acad. Sci. USA* **77,** 4623–4627.
7. Chang, K. J. & Cuatrecasas, P. (1979) *J. Biol. Chem.* **254,** 2610–2618.
8. Martin, W. R., Eades, C. B., Thompson, J. A., Huppler, R. E. & Gilbert, P. E. (1976) *J. Pharmacol. Exp. Ther.* **197,** 517–532.
9. Law, P. Y. & Loh, H. H. (1978) *Res. Commun. Chem. Pathol. Pharmacol.* **21,** 409–433.

10. Bowen, W. O., Gentleman, J., Herkenhan, M. & Pert, C. B. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 4818–4822.
11. Rothman, R. B. & Westfall, R. C. (1982) *Mol. Pharmacol.* **21,** 538–547.
12. Simonds, W. F., Terrence, R., Burke, T., Jr., Kenner, C., Jacobson, A. E. & Klee, W. A. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 4974–4978.
13. Bidlack, J. M., Abood, L. G., Osei-Gyimah, P. & Archer, S. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 636–639.
14. Gioannini, T., Foucaud, B., Hiller, J. M., Hatten, M. E. & Simon, E. J. (1982) *Biochem. Biophys. Res. Commun.* **105,** 1120–1125.
15. Manekjee, R., Zukin, R. S., Archer, S., Michael, J. & Osei-Gyimah, P. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 594–598.
16. Cho, T. M., Ge, B. L. & Loh, H. H. (1985) *Life Sci.* **36,** 1075–1085.
17. Cho, T. M., Ge, B. L., Yamato, C., Smith, A. P. & Loh, H. H. (1983) *Proc. Natl. Acad. Sci. USA* **80,** 5176–5180.
18. Pasternak, G. W. & Snyder, S. H. (1975) *Mol. Pharmacol.* **10,** 183–193.
19. Law, P. Y., Fischer, G., Loh, H. H. & Herz, A. (1979) *Biochem. Pharmacol.* **28,** 2257–2262.
20. Law, P. Y., Griffin, M. T., Koehler, J. E. & Loh, H. H. (1983) *J. Neurochem.* **40,** 267–275.
21. Loh, H. H., Cho, T. M., Wu, Y. C. & Way, E. L. (1974) *Life Sci.* **14,** 2231–2245.
22. Maizel, J. V., Jr. (1979) in *Methods in Virology*, ed. Maramorosch, K. & Koprowshi, H. (Academic, New York), Vol. 5, pp. 179–246.
23. Lindstrom, J., Lennon, V., Seybold, M. & Whittingham, S. (1976) *Ann. N. Y. Acad. Sci.* **274,** 254–274.
24. Laemmli, U. (1970) *Nature (London)* **227,** 680–685.
25. Lowry, O. H., Rosebrough, A. L., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193,** 265–275.
26. Schultz, R. M., Bleil, J. D. & Wasserman, P. M. (1978) *Anal. Biochem.* **91,** 354–356.
27. Newman, E. L. & Barnard, E. A. (1984) *Biochemistry* **23,** 5385–5389.
28. Howard, A. D., de La Baume, S., Gioaninni, T. L., Hiller, J. M. & Simon, E. J. (1985) *J. Biol. Chem.* **260,** 10833–10838.
29. Nozaki, M. & Cho, T. M. (1985) *J. Neurochem.* **45,** 461–464.
30. Sterweis, P. C. & Robishaw, J. (1984) *J. Biol. Chem.* **259,** 13806–13813.