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10. Poly-α-cyanoglycine (**8**) was synthesized by the base-catalyzed polymerization of α-cyanoglycine *N*-carboxyanhydride (**9**) (*8*). In a representative procedure carried out in a nitrogen dry box, a solution of **9** (4.5 g) in acetonitrile (50 ml) was stirred for 30 days at room temperature with two drops of a 1 percent solution of water in acetonitrile (*9*). A light brown precipitate formed that was filtered through fritted glass and dried in vacuo to yield a pale yellow powder (3.0 g). The weight average molecular weight determined from gel permeation chromatography was estimated at about 1300, corresponding to some 16 monomer units. Characteristic spectra show absorptions at 2245 cm^{-1} and 2200 cm^{-1} (infrared, $-C \equiv N$) and at 197 to 200 nm (ultraviolet).
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13. Hydrolysis of **8** yielded not only glycine but traces of alanine and aspartic acid, presumably formed from HCN eliminated during the synthesis of **8** from **9**. This suggests that in our experiment with $H^{13}CN$ and **8** exchange of nitrile groups might take place and could be one source of label in the amino acid products.
14. The material that was removed in vacuo was allowed to stand for 5 weeks when a tan polymer of hydrogen cyanide formed. On hydrolysis, this yielded glycine, alanine, valine, aspartic acid, and other compounds rich in ^{13}C (80 to 90 percent), showing that polymerization of this enriched HCN (90 percent $H^{13}CN$, 10 percent $H^{13}CN$) could be a source of label in the reaction with **8**.
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16. All mass spectral data were obtained on a model 3200 Finnigan gas chromatograph–mass spectrometer coupled with a model 6000 data system. The range m/e 35 to 300 was scanned approximately once per second and all data were stored on magnetic disc. Peak intensities were derived from single spectra after background subtraction, or in the case of low intensity peaks from the area of the reconstructed mass chromatogram for a particular ion. Gas chromatography conditions are described in (*15*).
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18. Structures **8** and **10** are precursors of glycine; **11**, of diaminosuccinic acid; and **12**, of aspartic acid and, perhaps alanine. Detailed mechanisms now being elucidated for side chain modification of **8**, particularly the steps involving the use of HCN as a reducing agent, are consistent with these labeling results as well as with the results obtained by deuterolysis of **8** with DCl-D_2O before and after HCN modification. We have found that for the amino acids derived from **8** by deuterolysis all hydrogen atoms attached to carbon atoms can be replaced by deuterium.
19. S. Akabori and M. Yamamoto [in *Molecular Evolution, Prebiological and Biological*, D. L. Rohlfing and A. I. Oparin, Eds. (Plenum, New York, 1972), p. 189] restate Akabori's 1953 proposal that the original heteropolypeptides on the earth were formed by polymerization of aminoacetonitrile to a polyamidine "fore-protein" which was hydrolyzed to polyglycine and then modified by aldehydes, and the like, to heteropolypeptides. We consider this model improbable because of the instability of aminoacetonitrile and the inertness of polyglycine. Diglycine and triglycine reported from attempts to polymerize aminoacetonitrile most probably were formed instead from eliminated hydrogen cyanide (*6*). We have shown that deuterolysis of **8** with DCl-D_2O yields glycine containing two atoms of deuterium per molecule more than glycine obtained by deuterolysis of polyglycine. This striking demonstration of the reactivity of the α-hydrogen and α-cyano groups of **8** suggests that its polyamidine analog **4**, derived solely from HCN, is a far more likely precursor of proteins than polyglycine or the fore-protein of Akabori.
20. We thank S. Kammeyer, R. Widing, and Dr. C. Warren for experimental help.

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Solubilization of a Stereospecific Opiate-Macromolecular Complex from Rat Brain

Abstract. *A [^{3}H]etorphine-macromolecular complex has been solubilized from rat brain synaptosomal fraction by extraction with the nonionic detergent Brij 36T. Stereospecificity of binding to this solubilized complex was demonstrated by the finding that radioactivity in the complex was virtually eliminated when binding had occurred in the presence of excess levorphanol, an active narcotic analgesic, while it was unaffected by its inactive enantiomorph dextrorphan. Bound radioactivity was dissociated by proteolytic enzymes, sulfhydryl reagents, and heat, suggesting the presence of protein. The bound solubilized macromolecular moiety may be the opiate receptor.*

The existence of stereospecific opiate binding sites in animal brain was discovered independently about 2 years ago in three laboratories, including our own (*1, 2*). Since then much has been learned about the properties of these binding sites by studying opiate binding in membrane preparations derived from brain homogenate. The observed properties are consistent with the hypothesis that these sites represent pharmacological opiate receptors. The exact chemical structure of these receptors and many aspects of receptor-drug interaction can only be learned when receptor molecules are available in soluble and highly purified form. As a first step toward this end we report the solubilization of etorphine (*3*) bound stereospecifically to a macromolecular moiety and present data suggesting that this moiety may be the opiate receptor.

The brains of Sprague-Dawley rats, after removal of the cerebella, were homogenized and used to prepare mitochondrial-synaptosomal (P_2) fractions (*4*). The P_2 fraction, resuspended in 0.32*M* sucrose, was diluted five-fold with 0.05*M* tris buffer, *p*H 7.4, to give the appropriate tissue concentration of an osmotically lysed membrane preparation. Prior to extraction with detergent, the P_2 membranes were incubated with [^{3}H]etorphine at 37°C for 20 minutes. As described earlier (*2, 4*), incubations were carried out in the presence of the active narcotic analgesic levorphanol (10^{-6}*M*) or in an equal concentration of its inactive enantiomorph dextrorphan, in order to establish the degree of stereospecificity of the etorphine binding (*5*). The membrane fraction was then centrifuged at 20,000*g* for 15 minutes at 4°C. The resulting pellet was resuspended in one-tenth the original volume of an ice-cold 1 percent solution of the nonionic detergent Brij 36T (Emulsion Engineering, Elk Grove Village, Ill.) in 0.01*M* tris buffer, *p*H 7.4, containing 0.2 m*M* dithiothreitol and 1 m*M* EDTA (*6*). This suspension was mixed briefly on a Vortex vibrator and immediately centrifuged in a Beckman preparative ultracentrifuge at 100,000*g* for 90 minutes. Free and bound etorphine in the supernatant were separated by passage through a column of XAD-4 Amberlite and elution with cold 0.05*M* tris, *p*H 7.4. Fractions (1 ml) were collected, and radioactivity was determined by counting portions in Aquasol in a liquid scintillation spectrometer. Protein concentration was determined by the method of Lowry *et al.* (*7*).

Free [^{3}H]etorphine adheres firmly and quantitatively (> 99 percent) to the XAD-4 column and can only be eluted with methanol or ethanol. Figure 1 shows another control in which [^{3}H]etorphine was added to a Brij extract of P_2 membranes (we previously ascertained that no binding occurs in the presence of 1 percent Brij). When the supernatant from ultracentrifugation of the extract was passed through a column of XAD-4, the bulk of protein (> 85 percent) appeared in the void volume, while the amount of radioactivity eluted was less than 1 percent.

When [^{3}H]etorphine was first bound to P_2 membranes in the presence of a 1000-

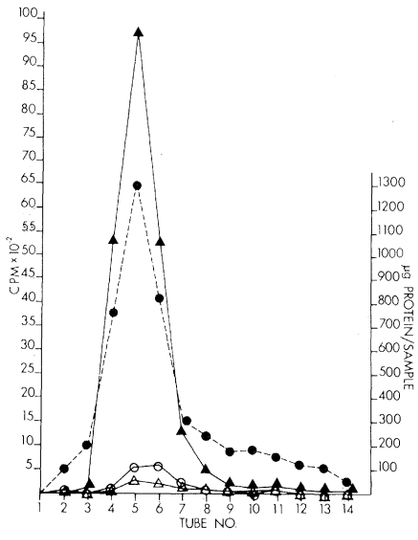

Fig. 1. Elution profile on XAD-4 of Brij extract of P_2 membranes bound with [^{3}H]etorphine. P_2 membranes (2 mg of protein per milliliter) were incubated with [^{3}H]etorphine ($1 \times 10^{-9}M$, 20.7 c/mmole) and subsequently extracted with 1 percent Brij 36T. A 1-ml sample of the supernatant, after ultracentrifugation, was placed on a column (2 by 10 cm) of XAD-4 (Rohm and Haas) and eluted with cold 0.05M tris buffer. (▲) [^{3}H]Etorphine bound in the presence of $10^{-6}M$ dextrorphan. (○) [^{3}H]Etorphine bound in the presence of $10^{-6}M$ levorphanol. (△) [^{3}H]Etorphine added to Brij extract of P_2 membranes subsequent to extraction and ultracentrifugation. (●) Protein concentration is given in micrograms per milliliter.

fold excess of dextrorphan and then extracted with detergent and ultracentrifuged, chromatography of the resultant supernatant on XAD-4 produced a peak of radioactivity coincident with that of protein. In a group of similar experiments this radioactive peak represented 25 to 30 percent of the total radioactivity in the supernatant. When the identical experiment was performed in the presence of a 1000-fold excess of levorphanol, the amount of radioactivity eluted was negligible. This latter experiment demonstrates the stereospecificity of the etorphine-bound complex solubilized by the detergent (Fig. 1).

Prior to these experiments we tested a large number of nonionic and ionic detergents for their ability to solubilize a labeled complex. Included were NP40, Lubrol, digitoxin, Triton X-100, Brij 35, Brij 56, sodium deoxycholate, and sodium dodecyl sulfate; all of these detergents solubilized most of the membrane-bound radioactivity. However, the supernatant radioactivity was found to be associated with a small-sized molecule, probably free etorphine. Only deoxycholate gave rise to a small yield of bound radioactivity (nonsedimentable at 20,000g) which was sedimented at 100,000g in 1 hour.

The solubilized material is quite stable at 0°C and can be dialyzed in the cold overnight with only minimal loss of radioactivity. At 37°C the solubilized bound material has a half-life of dissociation of only 10 minutes, compared to the dissociation rate for intact etorphine-bound membranes, which is about 45 minutes.

In an effort to determine whether the etorphine-bound moiety contains protein, we incubated the bound ultracentrifugal supernatant with the sulfhydryl reagent N-ethylmaleimide (NEM) or with proteolytic enzymes. Incubation at 37°C for 30 minutes with $10^{-3}M$ NEM reduced bound radioactivity by 90 percent, while trypsin (1 mg/ml) or Pronase (60 µg/ml) reduced bound ligand by 60 percent in 10 minutes at 25°C as compared to controls incubated under similar conditions. Heating at 50°C for 10 minutes resulted in 84 percent loss of bound radioactivity.

When the ultracentrifugal supernatant of a Brij extract of etorphine-bound membranes was placed on a Sepharose 6B column, about 25 to 30 percent of the radioactivity was eluted in a peak at an elution volume consistent with binding to a macromolecule, while the bulk of the radioactivity eluted at the same volume as authentic free etorphine. Chromatography of the extract on a Sepharose 6B column calibrated with proteins of known molecular weight (Fig. 2) yielded an estimate of about 370,000 daltons for the [^{3}H]etorphine-bound macromolecular complex.

Thus it is possible to solubilize an opiate-bound macromolecular moiety from membranes derived from rat brain by the use of the nonionic detergent Brij 36T. The criterion of solubilization that we use is nonsedimentation at 100,000g in 90 minutes. The solubilized bound complex has properties consistent with its being an etorphine receptor site complex. The binding exhibits high affinity and stereospecificity. In addition, binding is sensitive to proteolytic enzymes, a sulfhydryl reagent, and heat, suggesting the presence of protein. These results are similar to those obtained with intact membranes, except that there is a decreased sensitivity to proteolytic enzymes and a decrease in affinity, as shown by an increase in dissociation rate.

To date we have been unable to demonstrate binding of opiates to unbound or dissociated solubilized macromolecular material, a difficulty encountered by others in their attempts to solubilize receptors (*8*). This could be due to the presence of inhibitory concentration of detergent. A large portion of the detergent seems to be removed by the XAD-4 column, but we have no quantitative assay for Brij and therefore do not know its residual level in the eluate. The reduced affinity for etorphine binding may point to some distortion of the binding site. This may result in good retention of an already bound drug, but inability to rebind.

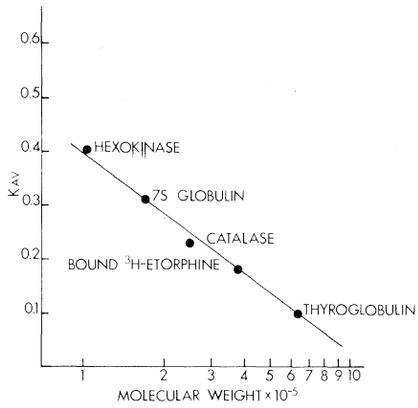

Fig. 2. Estimation of the molecular weight of solubilized [^{3}H]etorphine-bound complex on Sepharose 6B. Gel filtration was carried out on a column, 1 by 52 cm; the eluting solution was 0.05M tris buffer, pH 7.4. Data are expressed as K_{av} (*9*); by definition $K_{av} = (V_e - V_o/V_t - V_o)$, where V_e isthe elution volume corresponding to the peak concentration of solute (marker proteins monitored by absorbance at 280 nm, [^{3}H]etorphine-bound complex monitored by radioactivity determination), V_o is the void volume as determined by the appearance of dextran blue, and V_t is the total liquid volume as determined with free [^{3}H]etorphine. V_o and V_t values were 20 and 65 ml, respectively. The relation between the logarithm of the molecular weight and K_{av} was used to obtain the molecular weight of the [^{3}H]etorphine-bound complex.

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