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Competitive inhibition of benzodiazepine binding by fractions from porcine brain

(endogenous diazepam/psychotropic drugs)

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ABSTRACT **Fractions of porcine cerebral cortex extract separated by molecular weight on a Sephadex G-75 column were tested for their activities and potencies to inhibit [^{3}H]benzodiazepine binding to rat brain homogenates. The fractions spanned molecular weights from 500 to 100,000. A potent inhibitor (benzodiazepine-competitive factor I, BCF-I) was discovered in the fraction containing substances with molecular weights from 40,000 to 70,000. Equilibrium binding studies indicated that BCF-I was a competitive inhibitor, making it a candidate as a benzodiazepine endogenous factor or profactor. BCF-I was heat stable, but trypsin digestion destroyed its activity. Another inhibitory fraction (BCF-II) was 1/5th as active as BCF-I and contained substances with molecular weights from 1000 to 2000.**

It is an accepted pharmacologic concept that many drugs initially act by binding to effector cell membranes at specific receptor sites. In the brain many of the specific receptor sites are thought to be part of a system that depends on a neurohormone that binds to and activates these specific receptors. "Neurohormone" is used in an all-inclusive sense to mean any grain substance that is released and subsequently alters neural activity. If the benzodiazepines exert their effects by mimicking such a neurohormone, then it should be possible to demonstrate the binding of a radiolabeled benzodiazepine to brain homogenates containing some of the neurohormone's receptors. Workers in at least six independent laboratories have reported [^{3}H]benzodiazepine binding to brain homogenates (1–6), and all have reported a single specific binding site with nonspecific binding of only 10% or less. However, demonstration of binding is not sufficient proof that the binding site is a neurohormone receptor.

Other data lend support to the hypothesis that the benzodiazepine binding site is a neurohormone receptor, possibly located at a synapse. In summary, the binding site displays: high affinity (1), reversibility (6), stereospecificity (7), ligand specificity (4, 7–10), species specificity (11), organ specificity (1), synaptic localization (12), and increases in specific binding after preincubation, indicating the possible dissociation of an endogenous ligand (2, 13, 14).

In addition, a large number of nonbenzodiazepine drugs and neurohormones have been shown not to compete for the binding site (7, 10). Also, the distribution of benzodiazepine binding in the brain does not parallel the distribution of binding of any known drug or neurohormone (1). Such results indicate that the benzodiazepine binding site is not a receptor for these other substances but leave open the possibility that the benzodiazepines act on an undiscovered neurohormone receptor.

If the benzodiazepines are interacting with a neurohormone receptor system, it should be possible to extract the neurohormone from the brain and demonstrate its competitive inhibition of [^{3}H]benzodiazepine binding. Marangos *et al.* (15) and Karobath *et al.* (16) have examined this possibility by using acidified acetone extracts, minimizing molecules above 2000 daltons. Both groups reported an inhibitory entity of around 500 daltons, which was not affected by proteolytic enzymes. Marangos *et al.* demonstrated kinetically that their entity was a competitive inhibitor. Karobath *et al.* found that muscle extracts contained the highest activity of all tissues tested, but Marangos *et al.* reported the lowest activity in muscle.

We have examined porcine brain fractions that could contain entities between 500 and 100,000 M_r for competitive inhibition of specific benzodiazepine binding in rat brain subfractions; we found inhibition by both high and low M_r fractions. The high M_r (40,000–70,000) fraction [termed benzodiazepine-competitive factor I (BCF-I)] was five times more potent than the low M_r (1000–3000) fraction (BCF-II). The designations BCF-I and II were chosen because the entities compete with benzodiazepines at their binding site.

METHODS AND MATERIALS

Extraction and Fractionation of Porcine Cortex. Because the densities of benzodiazepine-specific binding sites have been reported to be highest in the cortex, cortex was expected to give the highest yield of endogenous benzodiazepine(s). For extraction, lyophilized porcine cortex (40 g) was homogenized at 4°C in a Waring Blendor in 500 ml of 2 M acetic acid/methanol (1:1, vol/vol) that contained 0.001% phenylmethylsulfonyl fluoride and 0.01% thiodiglycol (17). The mixture was centrifuged at 12,000 × *g* for 20 min at 4°C. The pellet was resuspended in the extraction solvents, homogenized in a Sorvall homogenizer, stirred for 12 hr at 4°C, and centrifuged. This procedure was repeated 3 times. The supernatants were combined, flash evaporated or lyophilized, redissolved in 1 M acetic acid, and defatted three times with hexane/ethyl acetate (3:1, vol/vol). The organic layer was washed three times with 1 M acetic acid and the combined aqueous layers were finally lyophilized to give the material for column purification.

The lyophilized material was purified on a 2.5 × 90 cm Sephadex G-75 column. The elution was with 1 M acetic acid that contained 0.01% thiodiglycol and 0.0001% pentachlorophenol (17); 20-ml fractions were collected.

Hydrolysis and Trypsin Digestion of Active Brain Fractions. The hydrolysis of the active fractions was carried out with 6 M HCl according to Stuart and Young (18). Trypsin digestion was carried out in 0.1 M K phosphate buffer, pH 7.5, by incubation with active fraction at 25°C for 60 min. The trypsin was

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Abbreviations: BCF, benzodiazepine-competitive factor; NaDodSO$_4$, sodium dodecyl sulfate.

subsequently inactivated by incubating at 100°C for 15 min before addition to the binding assay. This heat treatment did not affect the activity of undigested brain fractions.

Binding Assay. Sprague–Dawley rats (300–400 g) were decapitated and the brain (minus the cerebellum) was rapidly removed. The brains were homogenized in 10 vol of ice-cold 0.1 M K phosphate buffer, pH 7.05. The homogenate was centrifuged at 500 × *g* for 10 min to remove large particulate matter. The 500 × *g* supernatant was centrifuged at 12,000 × *g*. The 12,000 × *g* pellet was resuspended in the same phosphate buffer to a protein concentration of 1 mg/ml, determined according to Lowry *et al.* (19). This homogenate was incubated at 37°C for 30 min to dissociate any potential endogenous ligands and frozen at −20°C for use the following day.

Brain extracts were screened for inhibition of benzodiazepine binding by incubating them with either 5 nM [^{3}H]diazepam or 2 nM [^{3}H]flunitrazepam. One to ten mg of lyophilized brain extract was dissolved in 300 μl of 0.05 M acetic acid. Fifty microliters of the resulting extract solution was incubated at 0°C for 60 min with 50 μl of either 60 nM [^{3}H]diazepam (5 nM final) or 25 nM [^{3}H]flunitrazepam (2 nM final) and 500 μl of the brain homogenate (600 μl total volume).

Bound [^{3}H]benzodiazepine was determined by filtration of the binding assay reaction materials through Whatman GF/A filters. The reaction materials were washed out of the assay tubes with two 5-ml washed of ice-cold phosphate buffer, and the residues on the filters were washed twice with 5 ml of buffer. The filters were placed in 5 ml of Bray's solution and radioactivity was measured with a Prias liquid scintillation counter (Packard Instruments). Counting efficiency was 301%.

Specific binding was calculated by subtracting the nonspecific cpm from the total cpm. Nonspecific binding was estimated by incubating a labeled benzodiazepine with a 500-fold excess of the corresponding unlabeled benzodiazepine. Control binding was determined with 0.05 M acetic acid (the brain extract solvent), which shifted the final pH from 7.05 to 7.0, the optimal binding pH in this system (see *Results*). Inhibition of specific benzodiazepine binding was expressed as percent of control binding divided by the mg of lyophilized brain extract introduced into the final assay mixture. Equilibrium binding analyses were performed with various concentrations of [^{3}H]flunitrazepam in the above assay. Up to 0.5% ethanol was utilized in the final assay mixture to ensure that the higher concentrations of benzodiazepines stayed in solution. This amount of ethanol was earlier found not to affect binding significantly (13).

Sodium Dodecyl Sulfate ($NaDodSO_4$)/Polyacrylamide Gel Electrophoresis. Electrophoresis of brain extracts was performed in slab gels (20) with Laemmli's buffer system (21). The following modifications were made. Proteins were stained with 0.2% Coomassie brilliant blue R250 in a solution of methanol/H_2O/acetic acid (7:7:1, vol/vol). The gels were destained in 7% acetic acid. The slabs contained 10% acrylamide as the separating gel and 5% acrylamide as the stacking gel.

Materials. [*Methyl*-^{3}H]diazepam and [*methyl*-^{3}H]flunitrazepam were obtained from New England Nuclear and had specific activities of 40 Ci/mmol and 87.5 Ci/mmol, respectively (1 Ci = 3.7 × 10^{10} becquerels). Pure unlabeled diazepam and flunitrazepam were gifts of Hoffmann–La Roche.

RESULTS

pH Effects. Fig. 1 shows the effects of pH on specific diazepam binding. Maximal binding was obtained at pH 7.0, but binding was reduced to 50% of maximum at pH 6.0. Because pH is so critical for binding levels, 0.1 M phosphate buffer was used to screen brain extracts. This buffer, when compared to other buffers tested, had the highest buffering capacity at pH 7.0 and gave maximal binding levels. It was found that some brain fractions did not readily dissolve in this phosphate buffer, so they were initially dissolved in 0.05 M acetic acid before addition to the final assay. The small effect of this acid addition on pH was compensated for in the binding assay (see *Methods and Materials*).

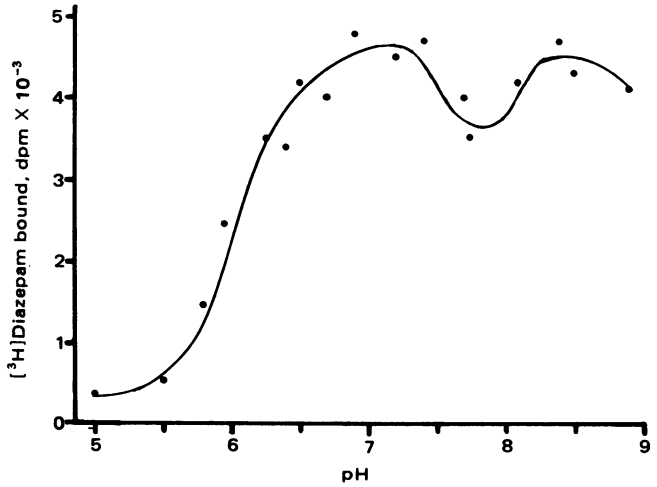

FIG. 1. Effect of pH on specific binding of 5 nM [^{3}H]diazepam in 0.1 M phosphate buffer. Standard assay conditions were used at various final pH values.

Inhibition of Diazepam Binding by BCF-I and BCF-II. Fig. 2 shows the inhibitory potency of a typical series of Sephadex G-75 fractions. The 30,000–70,000 M_r fraction (BCF-I) and the 1000–2000 M_r fraction (BCF-II) consistently contained the most diazepam binding inhibitory activity. BCF-I was 5 times more potent than BCF-II on a weight basis.

$NaDodSO_4$/Polyacrylamide Gel Electrophoretic Analysis of the Brain Fractions. Fig. 3 shows a $NaDodSO_4$/polyacrylamide gel electrophoretic analysis of some of the brain extract Sephadex fractions. Lanes A through E show that the M_r values of the brain fractions as estimated by prestandardized Sephadex column flow rates (compounds of known M_r were used as standards) are approximately the same as the M_r values esti-

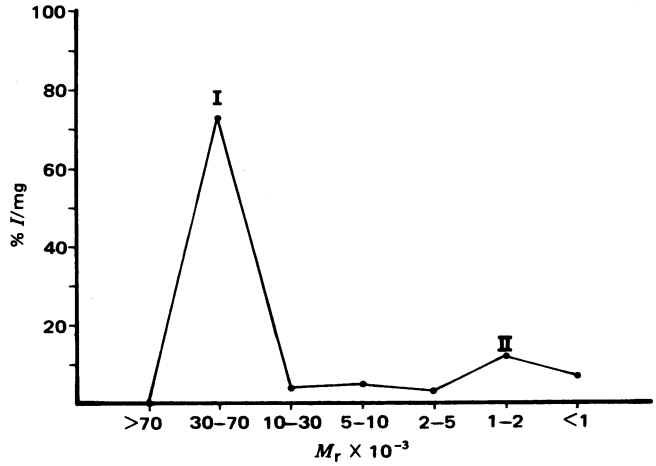

FIG. 2. The benzodiazepine binding inhibitory potency of a series of porcine brain extract fractions from a Sephadex G-75 column. M_r values were estimated by using prestandardized column flow rates. Potency was calculated as the percent inhibition of specific binding (% *I*) per mg of lyophilized extract fraction added to the final assay volume.

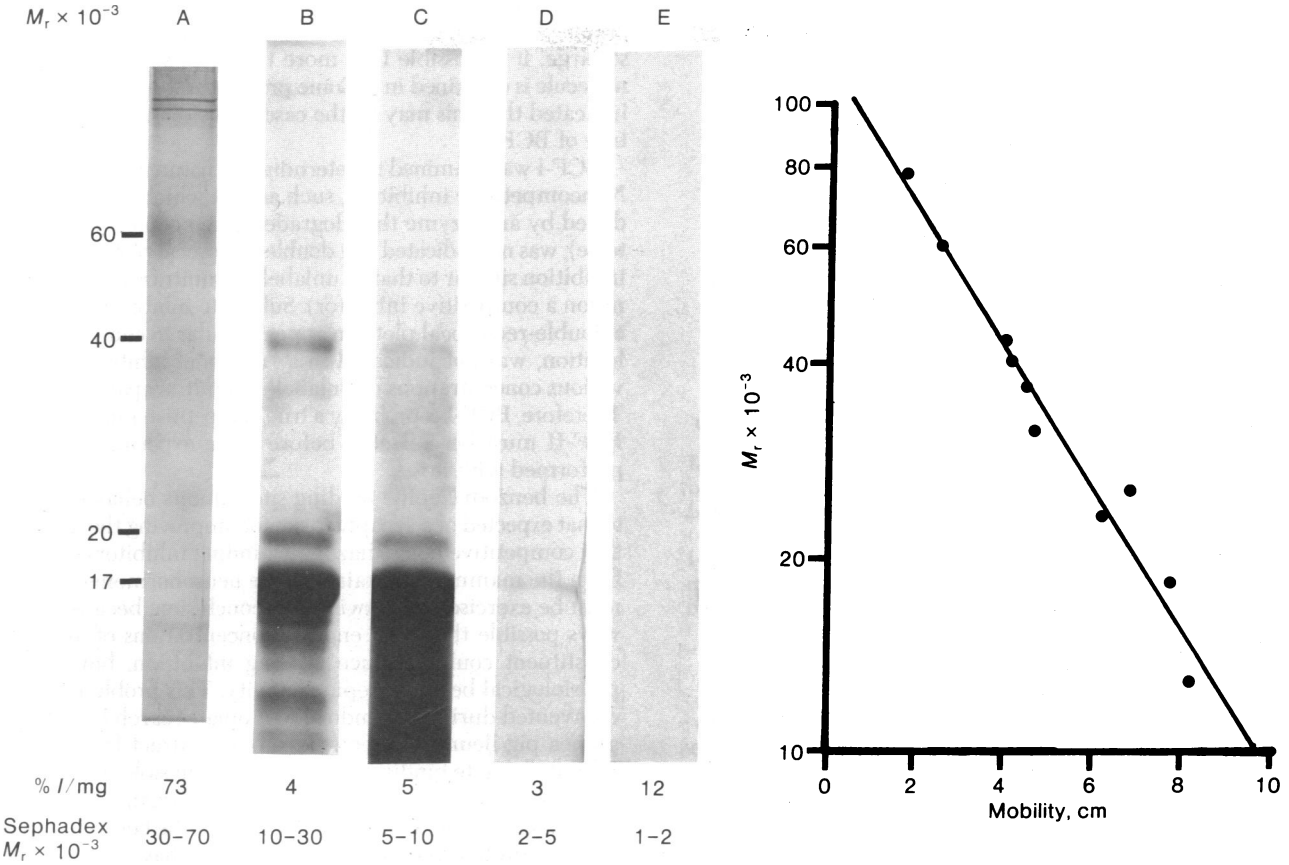

FIG. 3. (*Left*) NaDodSO$_4$/polyacrylamide gel electrophoresis of the porcine brain extract fractions shown in Fig. 2. (*Right*) Gel M_r values were determined by running a series of enzymes of known M_r as standards and plotting their M_r values on a logarithmic scale against their mobilities. Potencies and Sephadex M_r estimates of the fractions are as calculated in Fig. 2.

mated by NaDodSO$_4$ gel electrophoresis. BCF-I is shown in lane A of Fig. 3, and its major band corresponds to a protein of M_r 60,000. BCF-II is shown in lane E. The lack of bands indicates that all substances in BCF-II are below M_r 10,000. A portion of BCF-I was acid hydrolyzed. A gel analysis of the hydrolyzed sample revealed no bands at all, indicating all proteins had been hydrolyzed to small molecules.

Equilibrium Binding Analysis of BCF-I. Fig. 4 shows three double-reciprocal plots of the specific binding produced by various concentrations of labeled flunitrazepam in the presence or absence of 2 nM unlabeled flunitrazepam or 1 mg of BCF-I. The inhibition produced by BCF-I was nearly identical to that produced by 2 nM unlabeled flunitrazepam. This did not rule out substrate inhibition (inhibition by binding to the labeled flunitrazepam), which can appear similar to competitive inhibition. Fig. 5 shows S curves of the fractional inhibition (i) of specific binding produced by various concentrations (plotted as logarithms) of unlabeled flunitrazepam or BCF-I in the presence of 2 nM labeled flunitrazepam. The inhibition produced in the final assay by 1.0 mg of BCF-I was assigned as being equivalent to 2 nM unlabeled flunitrazepam on the basis of the data in Fig. 4. This causes the two semi-logarithmic S-shaped curves to overlap at the 2 nM unlabeled flunitrazepam point and allows their slopes to be compared. The *inset* of Fig. 5 shows the curves theoretically expected for this type of plot with a competitive inhibitor and a substrate inhibitor according to Webb (22). A substrate inhibitor should produce a steeper rise in i than a competitive inhibitor (22). The main plot in Fig. 5 shows that the BCF-I curve shows no hint of a steeper rise and is nearly identical to the unlabeled flunitrazepam curve. The highest concentration of BCF-I, which was near saturation, only produced a 70% ($i = 0.7$) inhibition of binding. Further purification should result in 100% inhibitory concentrations.

Thermal Stability, Trypsin Digestion, and Acid Hydrolysis of BCF-I. Table 1 shows that BCF-I is thermally stable, but it

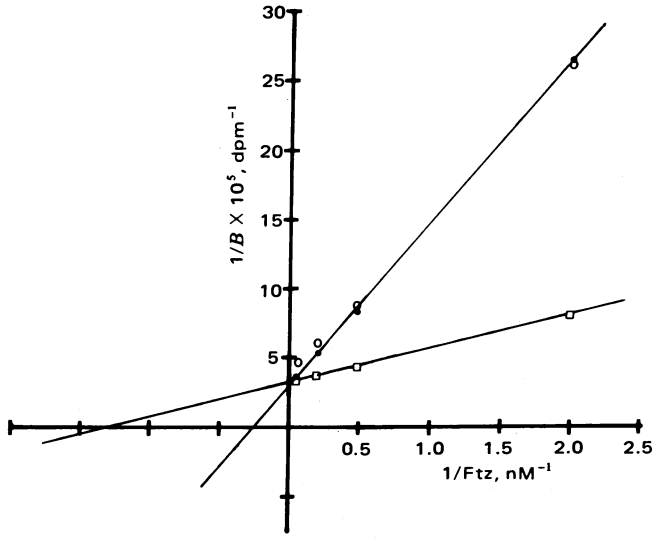

FIG. 4. Double-reciprocal plots of the specific binding (B) produced by various concentrations of [^{3}H]flunitrazepam (Ftz) in the presence or absence of inhibitors. □, Control; ●, 2 nM unlabeled flunitrazepam; ○, 1.0 mg BCF-I. Both unlabeled flunitrazepam and BCF-I were dissolved in 0.05 M acetic acid. Control is 0.05 M acetic acid only.

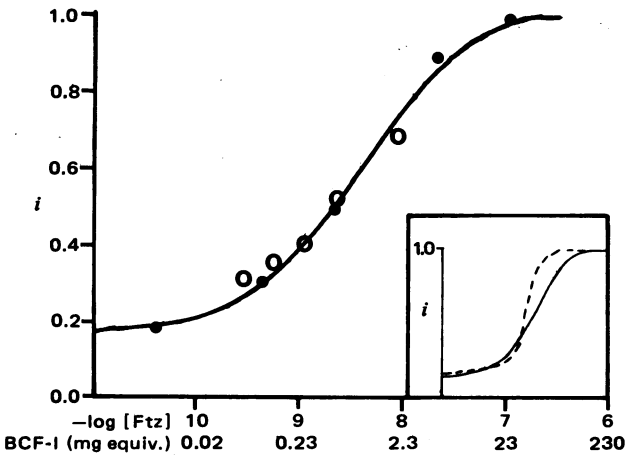

FIG. 5. Fractional inhibition (i) of the specific binding of 2 nM [^{3}H]flunitrazepam produced by various concentrations of unlabeled flunitrazepam [Ftz] (●) or BCF-I (○). *Inset,* Results that would theoretically be expected, if BCF-I were a substrate inhibitor (- - -) or a competitive inhibitor (—). BCF-I (mg equiv.) = mg of BCF-I added to the final assay, plotted on a logarithmic scale. One milligram of BCF-I was set as being equivalent in inhibition to 2 nM unlabeled flunitrazepam on the basis of Fig. 4.

loses its inhibitory activity after trypsin digestion or acid hydrolysis. While heating at 100°C for 15 min completely inactivated trypsin, it had no effect on nontrypsinized BCF-I. The loss of activity after trypsin digestion or acid hydrolysis indicates the presence of peptide bonds in the active portion of the BCF-I molecule(s).

Discussion. We have attempted to extract factors from porcine brain that competitively inhibit benzodiazepine binding. Inhibitory activity was found in both a high (BCF-I) and a low (BCF-II) M_r fraction from a series of brain extract fractions. BCF-I proved to be somewhat more potent than BCF-II and was studied further.

We tried to use a method of expressing inhibition of binding that was as accurate and universally applicable as possible. Percent inhibition of control binding, corrected for the milligrams of dry fractional residue added to the final binding assay, was used as the quantitative measure of inhibitory potency. Although inhibitor molarity would have been a better way to express potency it was felt that mistakes in M_r estimates would introduce too much error into molarity calculations.

Table 1. Effects of heat, trypsin digestion, or acid hydrolysis on BCF-I

Sample	Specific binding, % control*
Solvent control	100 ± 4
BCF-I† 0°C, 90 min	64 ± 1
25°C, 90 min	65 ± 3
100°C, 15 min	68 ± 4
Trypsin-digested BCF-I‡	96 ± 1
Acid-hydrolyzed BCF-I§	98 ± 2

* Mean ± SD of triplicates.
† BCF-I (0.5 mg) was incubated at the indicated temperature for the indicated time before addition to the binding assay.
‡ BCF-I (0.5 mg) was incubated with 2% trypsin at 25°C for 60 min followed by incubation at 100°C for 15 min to inactivate the trypsin before addition to the binding assay.
§ BCF-I (0.5 mg) was heated overnight at 100°C in 6 M HCl. Most of the HCl was then drawn off over NaOH crystals under reduced pressure. Any remaining HCl was carefully neutralized with dilute NaOH to pH 7.0 before addition to the binding assay.

According to gel electrophoresis, BCF-I contains mostly polypeptides from 30,000 to 70,000 M_r. Because this range is so large, it is possible that more than one size of inhibitory molecule is contained in it. Some preliminary experiments have indicated that this may be the case; this situation may also be true of BCF-II.

BCF-I was examined to determine the nature of its inhibition. Noncompetitive inhibition, such as that which would be produced by an enzyme that degraded the receptor (e.g., a protease), was not indicated in a double-reciprocal plot that showed inhibition similar to that of unlabeled flunitrazepam (by definition a competitive inhibitor). Substrate inhibition, which in a double-reciprocal plot can appear similar to competitive inhibition, was not indicated in a fractional inhibition plot of various concentrations of unlabeled flunitrazepam or BCF-I. Therefore, BCF-I is probably a true competitive inhibitor. More BCF-II must be collected before these experiments can be performed with it.

The benzodiazepine binding site exhibits behavior similar to that expected of a synaptic receptor, improving the likelihood that competitive benzodiazepine binding inhibitors extracted from the mammalian brain may be neurohormones. Caution must be exercised in drawing this conclusion, because it is always possible that high enough concentrations of any brain constituent could produce binding inhibition, but have no physiological benzodiazepine activity. This problem was circumvented during the endogenous opiate search by using the guinea pig ileum to screen those brain extract fractions that inhibited opiate binding for opiate-like physiological activity. Unfortunately, a highly specific sensitive organ bath bioassay like the guinea pig ileum does not exist for the benzodiazepines. This means that physiological testing will have to be done by using behavioral assays (such as protection against metrazol-induced convulsions), which will require relatively large amounts of purified inhibitor.

A molecule of M_r from 30,000 to 70,000 would be a neurohormone of unprecedented size. It is possible that such a large molecule may actually have a smaller active molecule as either part of its sequence or tightly bound to it in such a way that the activity of the smaller molecule is retained or enhanced. The larger molecule could function as a carrier or prohormone. Boiling BCF-I for 15 min did not diminish its activity. This indicates that tertiary structure is either very stable or absent. The active molecule(s) is probably a polypeptide, because trypsin digestion resulted in the loss of inhibitory activity. Molecules in the 30,000–100,000 M_r region that have opiate activity have recently been discovered in the brain (23). These large molecules can be broken down to form the more familiar endorphines and enkephalins, lending credence to the prohormone role postulated above for BCF-I. BCF-II falls into the M_r range of the endorphins, which are small enough to function as neurohormones.

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