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Inhibition of [^{3}H]diazepam binding by an endogenous fraction from rat brain synaptosomes

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Benzodiazepine derivatives are among the most widely used sedative agents in the treatment of anxiety, tension, alcoholism, epilepsy, and insomnia. Recently, they have been shown to bind to specific receptors in the brain (Squires & Braestrup 1977; Möhler & Okada 1977a, b). The search for endogenous compounds that exist in the brain, and bind to these receptors, has met with some success. Two groups have independently reported that inosine and hypoxanthine can act as competitive ligands for the diazepam receptor (Skolnick et al 1978; Asano & Spector 1979). Both of these purines, however, have a low affinity for the receptor in that millimolar quantities are required for 50% inhibition of [^{3}H]diazepam binding. This concentration would be difficult to achieve under physiological conditions and, consequently, their role as endogenous ligands is unclear. Two additional compounds have been isolated from brain and reported to alter diazepam binding when tested in this radioreceptor assay. The first compound was shown to be a 15 000 amu (unified) protein that modulates the affinity of both benzodiazepines and GABA for their respective receptors (Guidotti et al 1978). The second compound, a protein of 30 000–70 000 amu, was isolated from porcine brain (Colello et al 1978). Because of its unprecedented size as a receptor ligand, this protein could be a proneurohormone. Conclusions reached from the above research reports have led us to further examine brain tissue for a compound(s) that can serve as the natural 'occupant' of the diazepam binding sites. Since diazepam receptors are found in the crude synaptosomal fraction (Mackerer et al 1978), and since brain membranes, when prepared for in vitro use, apparently maintain endogenous ligands bound to their specific receptors (Williamson et al 1978; Pert & Bowie 1979; Lippa et al 1978; Davis & Ehrlich 1979), we have utilized these biological enrichments of 'occupied' binding sites as the source of material in the search for endogenous diazepam ligands.

For the preparation of naturally occurring ligands, the cerebrums of decapitated rats ($n = 5$) were homogenized at 4 °C in 20 volumes of ice cold 0·32 M sucrose and centrifuged at 10 000 *g* min. The resulting supernatant was then centrifuged at 300 000 *g* min. This crude synaptosomal (P_2) fraction was then lysed by suspension in 5 volumes of 50 mM Tris-Cl buffer (pH 7·5), shaken for 30 min in the cold (4 °C), and frozen at −80 °C. After thawing, the suspension was again shaken in the cold for 30 min and then centrifuged at 10^6 *g* min. The supernatant obtained after these incubations and the freeze-thawing cycle was then lyophilized. The dried residue was dissolved in 0·01 M acetic acid and fractionated by gel filtration on a BioGel (Bio-Rad, Richmond, California) P-10 column. Evaluation of the chromatographic separation of the soluble components from the lysed synaptosomal fraction demonstrated that four major peaks could be distinguished by ultraviolet absorption. The four peak fractions were freeze dried. The fractionated material consisted of components in the molecular weight range of 1000 amu to those in excess of 20 000 amu.

Aliquots of the original material applied to the gel filtration column and each of the reconstituted fractions were tested for their inhibitory potential in the [^{3}H]-diazepam binding assay. The binding assay was performed with [^{3}H]diazepam (a generous gift from New

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Table 1. Displacement of specific [^{3}H]diazepam from rat cerebral synaptosomal (P_2) membranes by endogenous factors.

	% inhibition*
Synaptosomal supernatant (original)	54·1
Gel filtration fractions	
I	24·1
II	25·9
III	28·4
IV	0·0
Ion-exchange fractionation of III	
III-A	5·9
III-B	9·4
III-C	82·4

* Average of duplicate determinations.

England Nuclear) according to the procedure of Möhler & Okada (1977b). Briefly, a P_2 pellet was prepared from rat cerebrum as described above. This synaptosomal fraction was rehomogenized in 15 volumes of hypotonic 50 mM Tris-Cl buffer (pH 7·5). Aliquots (300 μl) of the synaptosomal homogenate were added to plastic tubes in addition to 100 μl of either buffer, unlabelled diazepam (a kind gift from Hoffmann-LaRoche), or the fractions obtained from brain. To initiate the binding assay, 100 μl of [^{3}H]diazepam (1·6 nM) was added. After the incubation, the mixture was centrifuged and the resultant pellets were solubilized and counted by scintillation spectroscopy. This diazepam binding assay was shown to be saturable, time-dependent, reproducible, and specific in agreement with published reports (Möhler & Okada 1977a, 1977b; Squires & Braestrup 1977; Mackerer et al 1978; Williamson et al 1978).

The potency of inhibition for the endogenous material is shown in Table 1. Three fractions (estimated mol. wt: Fraction I, > 20 000; Fraction II, 10 000–20 000; Fraction III, 3000–6000) block [^{3}H]diazepam binding. A slight increase in potency was observed with decreasing molecular size. Whether these fractions of different sizes are related to each other in structure (like the relationship in the lipotropin-endorphin-enkephalin system) or whether these different factors exist as independent endogenous ligands (or modulators) of diazepam receptors awaits further investigation.

Initial experiments attempting to purify the active inhibitory component(s) present in Fraction III indicate that a single factor, which blocks [^{3}H]diazepam binding, can be obtained. Subjecting Fraction III to both anion- and cation-exchange chromatography resulted in three fractions: Fraction III-A was eluted from the Dowex 1-Cl^- column with 0·25 M HCl; Fraction III-B was eluted from the Dowex 50-H^+ column with 0·2 M NH_4OH; and Fraction III-C was recovered in the water wash as the fraction that did not bind to either column. When these three fractions (III-A, III-B, and III-C) were tested in the diazepam binding assay, only fraction III-C was found to be a potent inhibitor of [^{3}H]diazepam binding (Table 1). Evaluation of the components present in fraction III-C by thin layer chromatographic procedures on Silica Gel G plates (solvent butanol–acetic acid–ethyl acetate–water, 1 : 1 : 1 : 1) with substance identification by fluorescamine staining (Felix & Jimenez 1974) demonstrated the presence of a single component which we are currently characterizing. It has physical characteristics different from the previously reported endogenous diazepam ligands (Skolnick et al 1978; Guidotti et al 1978; Colello et al 1978; Asano & Spector 1979). Its isolation from membrane fractions enriched in benzodiazepine receptors (Mackerer et al 1978) might support its role as an endogenous ligand for this receptor. Other specific receptors occur in this fraction also. Thus, pharmacological determinations of the specificity of this endogenous factor for the benzodiazepine receptor in addition to biochemical, physiological and behavioural experiments must be performed before any conclusions can be drawn on whether the brain contains its own 'anti-anxiety' substance.

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