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Purification of a benzodiazepine from bovine brain and detection of benzodiazepine-like immunoreactivity in human brain

(benzodiazepine receptor/endogenous ligand/monoclonal antibody/agonist)

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ABSTRACT **An endogenous brain substance that binds to the central-type benzodiazepine receptors with agonist properties is present in both rat and bovine brains. This substance has been purified to homogeneity from bovine brain by immunoaffinity chromatography on immobilized monoclonal anti-benzodiazepine antibody followed by gel filtration on Sephadex G-25 and two reversed-phase HPLC steps. The purified substance was characterized as the benzodiazepine *N*-desmethyldiazepam (nordiazepam). The techniques used for the identification were mass spectrometry, HPLC, spectrophotometry, benzodiazepine receptor binding, and immunological techniques. Benzodiazepine-like immunoreactivity was also found in all the human brains tested, including six brains that had been stored in paraffin since 1940, fifteen years before the first synthesis of benzodiazepines. These results show that benzodiazepine-like molecules of natural origin—and possibly benzodiazepines themselves—are present in human and other mammalian brains.**

We have previously reported (1) the existence of endogenous benzodiazepine-like molecules in selected neuronal populations of rat brain. In these studies we used an anti-benzodiazepine monoclonal antibody (mAb) (1, 2). This finding was supported by immunocytochemistry and immunoblotting experiments as well as by the purification by immunoaffinity chromatography of a rat brain substance that inhibited [^{3}H]flunitrazepam binding to the neuronal-type benzodiazepine receptor (3, 4). The purified substance, however, did not inhibit the binding of [^{3}H]Ro 5-4864 to the peripheral-type benzodiazepine receptor. The immunoaffinity-purified substance blocked the binding of agonists, inverse agonists, and antagonists to the neuronal-type benzodiazepine receptor. The neurotransmitter γ-aminobutyric acid increased the affinity of the benzodiazepine receptor for the purified endogenous substance, which indicated that the purified substance is a benzodiazepine receptor agonist.

We also indicated (1) that the purified substance has properties different from those of the putative endogenous benzodiazepine receptor ligands reported in the literature, including among others the diazepam binding inhibitor (DBI) and related peptides (5–10). Whereas the DBI peptide binds to the β-carboline (inverse agonist) site of the benzodiazepine receptor and is anxiogenic and Pronase-sensitive, the substance that we have purified by immunoaffinity chromatography binds to the agonist binding site and is Pronase-resistant. These properties, together with the γ-aminobutyric acid effect, suggest that this is an anxiolytic substance.

We now describe the purification to homogeneity and the chemical identification of the substance as the benzodiazepine *N*-desmethyldiazepam. We have also found benzodiazepine-like immunoreactivity in human brains that had been stored in paraffin since 1940. Some of these results have been communicated in preliminary form (11–13).

MATERIALS AND METHODS

Materials. [^{3}H]Flunitrazepam (85 Ci/mmol; 1 Ci = 37 GBq) was from New England Nuclear. All the unlabeled benzodiazepines were gifts of R. I. Fryer, N. W. Gilman, W. E. Scott, and P. F. Sorter of Hoffmann-La Roche. Paraffin-embedded samples of human cerebella, stored since 1940, were kindly provided by H. Okazaki of the Mayo Clinic.

The anti-benzodiazepine mAb 21-7F9 was obtained after immunizing BALB/c mice with 3-hemisuccinyloxyclonazepam coupled to bovine serum albumin. The production, purification, and characterization of mAb 21-7F9 have been reported (2).

Immunoaffinity Chromatography. Fifty grams of bovine cerebellum and cerebral cortex were homogenized (Waring blender, 6 min) in 60 ml of 5 mM Tris·HCl, pH 8.1/0.3 mM phenylmethylsulfonyl fluoride/1 mM EDTA containing soybean trypsin inhibitor (20 μg/ml) and pepstatin (1 μg/ml). The preparation of the soluble extract and the procedure for immunoaffinity chromatography have been described (1). The sample was loaded on a 10-ml Affi-Gel 10 (Bio-Rad) column containing 50 mg of immobilized anti-benzodiazepine mAb 21-7F9, and 100 ml of each washing solution was passed through the column at a flow rate of 10 ml/hr. The retained material was eluted with 100 ml of 0.2 M acetic acid at a flow rate of 50 ml/hr.

Gel Filtration on Sephadex G-25. The immunoaffinity eluate was dried under reduced pressure, dissolved in 0.9 ml of 40% formic acid, and layered on a Sephadex G-25 column (51.5 cm × 1.2 cm i.d.) that had been equilibrated with 40% formic acid. Fractions (2 ml) were collected at a flow rate of 5 ml/hr. The active fractions were pooled, concentrated under reduced pressure, and resuspended in 600 μl of 0.1% trifluoroacetic acid. The benzodiazepine receptor binding activity of the fractions was assayed as indicated below.

Reversed-Phase HPLC. *Step I.* A 200-μl sample of the Sephadex G-25-purified substance was subjected to reversed-phase HPLC on a C_8 column [length, 25 cm; particle size, 10 μm; pore size, 300 Å; Brownlee Laboratory (Santa Clara, CA)] and eluted with a linear gradient (5–60%) of acetonitrile in 0.1% trifluoroacetic acid.

Step II. The active fractions from step I were pooled, dried under reduced pressure, and suspended in 150 μl of 0.1% trifluoroacetic acid. A 50-μl aliquot was applied to a 25-cm C_{18} Vydac column (5-μm particle size and 330-Å pore size). Elution was done with an exponential gradient (5–26%) of acetonitrile, using triethylammonium phosphate for ion-pairing [0.02048% (vol/vol) triethylamine and 0.0228%

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Abbreviations: DBI, benzodiazepine binding inhibitor; mAb, monoclonal antibody.

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(vol/vol) phosphoric acid in H_2O]. The flow rate for both HPLC systems was 1 ml/min, and fractions were collected every minute. The absorbance was monitored at 210 nm. After HPLC step II the fractions were applied to C_{18} Sep-Pak cartridges (Waters Associates) and eluted with 3 ml of isopropanol/0.1% trifluoroacetic acid (1:1). At the end of each purification step 200-μl aliquots of each fraction were dried under reduced pressure, dissolved in 200 μl of 50 mM Tris·HCl (pH 7.4), and tested for activity.

Receptor Binding Activity. Activity of the samples was determined by measuring the inhibition of [^{3}H]flunitrazepam binding to rat or bovine brain membrane benzodiazepine receptor, as described (1). The amount (μg) of purified substance was determined from the absorption at 235 nm related to a standard A_{235} curve generated by using known quantities of *N*-desmethyldiazepam.

Mass Spectra. Spectra were taken with an LKB-900 spectrometer in the electron-impact mode with 70-eV ionizing voltage and 20 μA ionizing current. The source was 270°C, and the sample was admitted by direct insertion probe at 100–200°C.

Immunocytochemistry. Paraffin-embedded blocks of human cerebella were sectioned (14 μm thick) and mounted on glass slides. The paraffin was removed and the tissue was hydrated by successive treatments (1 min each) with xylene (four times), 100% ethanol (twice), 95% ethanol (once), 70% ethanol (once), and H_2O (once). The mounted tissue was reacted with the mAb 21-7F9 according to a peroxidase-antiperoxidase procedure explained elsewhere (14, 15).

RESULTS

Purification. The immunoaffinity-purified substance migrated on Sephadex G-25 as a single peak with an elution volume that was larger (corresponding to smaller size) than that of cyanocobalamin (M_r 1355). The Sephadex G-25-purified substance was further purified to homogeneity by two sequential reversed-phase HPLC steps. In both HPLC steps, the inhibitory activity comigrated with the main A_{210} peak (Figs. 1 and 2). Further evidence of the homogeneity of the substance was that the absorption spectra of the various fractions forming the main peak of either Fig. 1 or Fig. 2 were identical.

Absorption Spectrum. Fig. 3 shows that the absorption spectrum of the purified substance is identical to that of *N*-desmethyldiazepam and that it is very similar to the spectrum of diazepam. However, the spectrum of the purified substance was very different from the spectra (not shown) of the other benzodiazepine receptor ligands tested, including chlordiazepoxide, medazepam, oxazepam, flunitrazepam, Ro 5-4864, Ro 15-1788, β-carbolines, purines, purine nucleosides, and nicotinamide among others. The purified substance showed three absorption peaks at 233, 280, and 360 nm in 0.1% trifluoroacetic acid/45% acetonitrile.

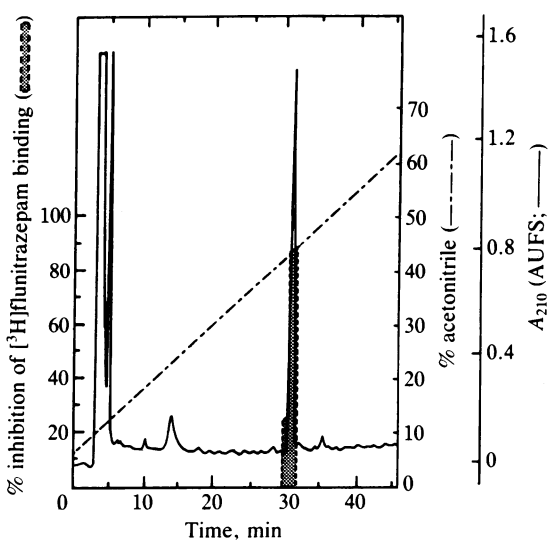

FIG. 1. Reversed-phase HPLC step I of the active substance. AUFS, absorbance units full-scale.

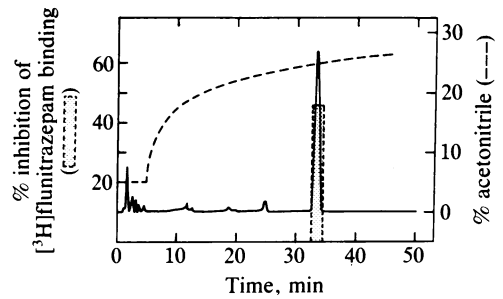

FIG. 2. Reversed-phase HPLC step II of the active substance obtained from HPLC step I (Fig. 1). Solid line represents A_{210}; maximum absorbance of predominant peak was 0.4 absorbance units full-scale.

Characterization of the Endogenous Substance. The purified substance comigrated with authentic *N*-desmethyldiazepam in reversed-phase HPLC. However, the mobility of the purified substance was different from that of diazepam (Fig. 4). When the purified substance was mixed with *N*-desmethyldiazepam, the two compounds migrated in a single peak in both HPLC systems. The mass spectrum of the purified substance was virtually identical to that of authentic *N*-desmethyldiazepam (Fig. 5). Useful spectra appeared as the probe was heated to 100°C, about the same temperature as required by authentic *N*-desmethyldiazepam. Thus the presence of a glycosidic or other functional link attached to the nitrogen in place of hydrogen is unlikely. The purified substance and *N*-desmethyldiazepam have also in common the following binding properties. (*i*) They have similar affinities for the neuronal-type benzodiazepine receptor, as determined from calculating the inhibition by the two substances of [^{3}H]flunitrazepam binding to the receptor ($IC_{50} \approx 2 \times 10^{-8}$ M). (*ii*) They have similar affinities for the mAb 21-7F9 (IC_{50} for [^{3}H]flunitrazepam binding to the mAb is $\approx 2 \times 10^{-7}$ M). (*iii*) They do not inhibit the binding of [^{3}H]Ro 5-4864 to the peripheral-type benzodiazepine receptor. (*iv*) γ-Aminobutyric acid potentiates to a similar extent the affinities of both substances for the benzodiazepine receptor.

Benzodiazepine-Like Immunoreactivity in Human Brains. Fig. 6 shows the distribution of the binding of mAb 21-7F9 to

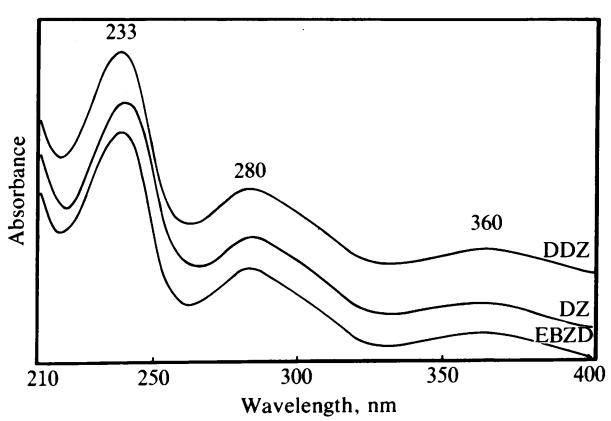

FIG. 3. Absorption spectra of the purified endogenous benzodiazepine (EBZD), *N*-desmethyldiazepam (DDZ), and diazepam (DZ).

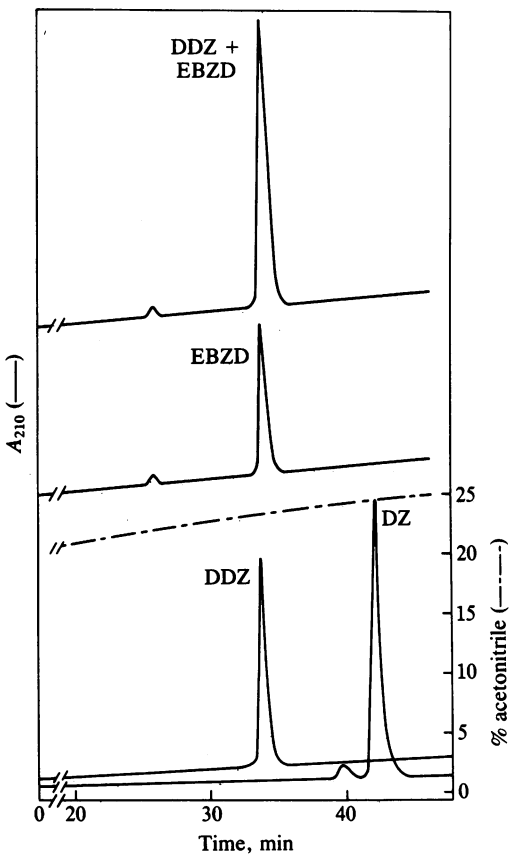

FIG. 4. Reversed-phase HPLC (step II system) of the purified endogenous benzodiazepine (EBZD), *N*-desmethyldiazepam (DDZ), and diazepam (DZ). Retention times were 35.29, 35.36, 35.36, and 42.91 min for EBZD, DDZ, EBZD + DDZ, and DZ, respectively.

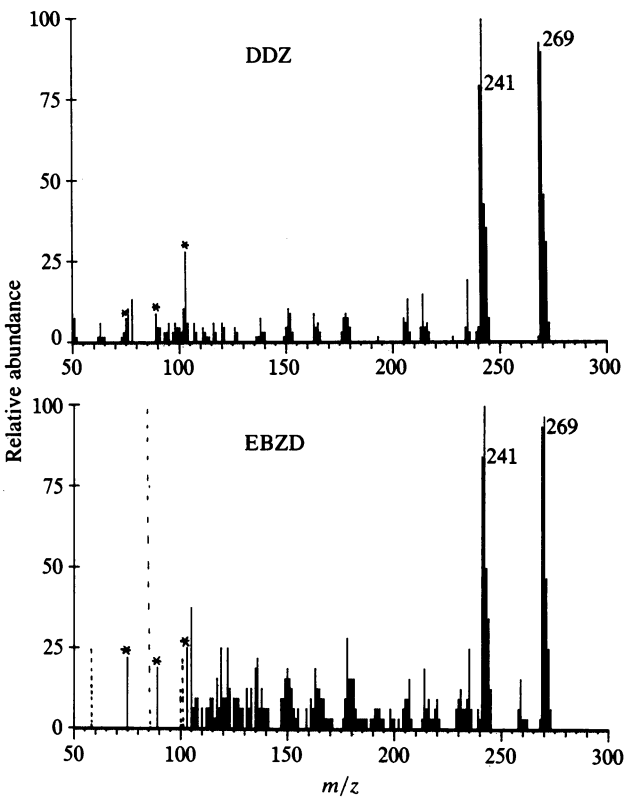

FIG. 5. Mass spectra of *N*-desmethyldiazepam (DDZ) and the endogenous benzodiazepine (EBZD). The ion peaks shown in dashed lines are probably due to the presence of triethylamine and other buffer components. For this reason, the peaks below *m/z* 102 are not very significant. Nevertheless, the doubly charged ions at *m/z* 75, 89, and 103 (labeled with asterisks) are present in both spectra.

an adult human cerebellum that had been stored in paraffin since 1940. The distribution of immunoreactivity is identical to that observed with fresh rat cerebellum fixed with periodate/lysine/4% paraformaldehyde (1). The benzodiazepine-like molecules are present in the perikarya and processes of all the major cerebellar neurons in both human and rat brains. The binding of mAb 21-7F9 to human cerebellum was blocked by ligands with high affinities for the mAb (0.1 μM diazepam, flunitrazepam, or Ro 5-4864) but it was not affected by 10 μM β-carboline-3-carboxylate ethyl ester or Ro 15-1788, which are ligands with low affinities for the mAb (2). The blockage of immunoreactivity by Ro 5-4864 and diazepam is due to the high affinities of these benzodiazepines for mAb 21-7F9 and does not necessarily reflect the specificity of the endogenous substance for the "peripheral" or "central" type of benzodiazepine receptor. We have observed identical benzodiazepine-like immunoreactivity in all five human (1 infant and 4 adult) cerebella tested; all five cerebella had been stored in paraffin since 1940.

DISCUSSION

We have purified the benzodiazepine *N*-desmethyldiazepam from bovine brain. [A molecule with similar properties (although not chemically characterized) has been isolated from rat brain (1).] The identification is based on immunological, biochemical, and pharmacological evidence. The yield of *N*-desmethyldiazepam from the richest bovine brain samples was 2.2 nmol/g of wet cerebellum and cortex, which indicates that the purified substance could be present in the brain at concentrations that could produce biological effects. However, the yield was not the same for all brain samples. The yield of activity from rat brains and some cattle brains was lower by a factor of 100. These quantitative differences might reflect differential exposure of the animals to environmental benzodiazepines. A second substance, with a different mobility in HPLC, was also detected in some preparations (data not shown). The activity and amount of this second substance was about 10% those of the *N*-desmethyldiazepam isolated. This second substance has not been completely characterized, but it has an absorption spectrum similar to that of the benzodiazepine oxazepam and has a mobility similar to authentic oxazepam in HPLC. Oxazepam is generated in mammals by hydroxylation of *N*-desmethyldiazepam. Manning and coworkers (16) have recently isolated *N*-desmethyldiazepam from human serum, using a purification procedure that did not involve antibodies. *N*-Desmethyldiazepam was also found in the sera of some of these authors who had never taken synthetic benzodiazepines. The authors could neither rule out nor demonstrate that the origin of the isolated benzodiazepine was industrial. Our findings are also consistent with those of the opiate system, where morphine and other nonpeptide opiates have been found in brain (17–19), adrenal gland (19), toad skin (20), and cow and human milk (21). Evidence for the biosynthesis of codeine and morphine in rats has been reported recently (27).

The purification of *N*-desmethyldiazepam from brain was unexpected. We were initially looking for endogenous peptides with benzodiazepine-like action. The main question that needs to be answered is whether the brain benzodiazepines are endogenous or exogenous and what is their biological significance. For this purpose, biosynthesis experiments are needed. We feel that it is unlikely that a heterocyclic

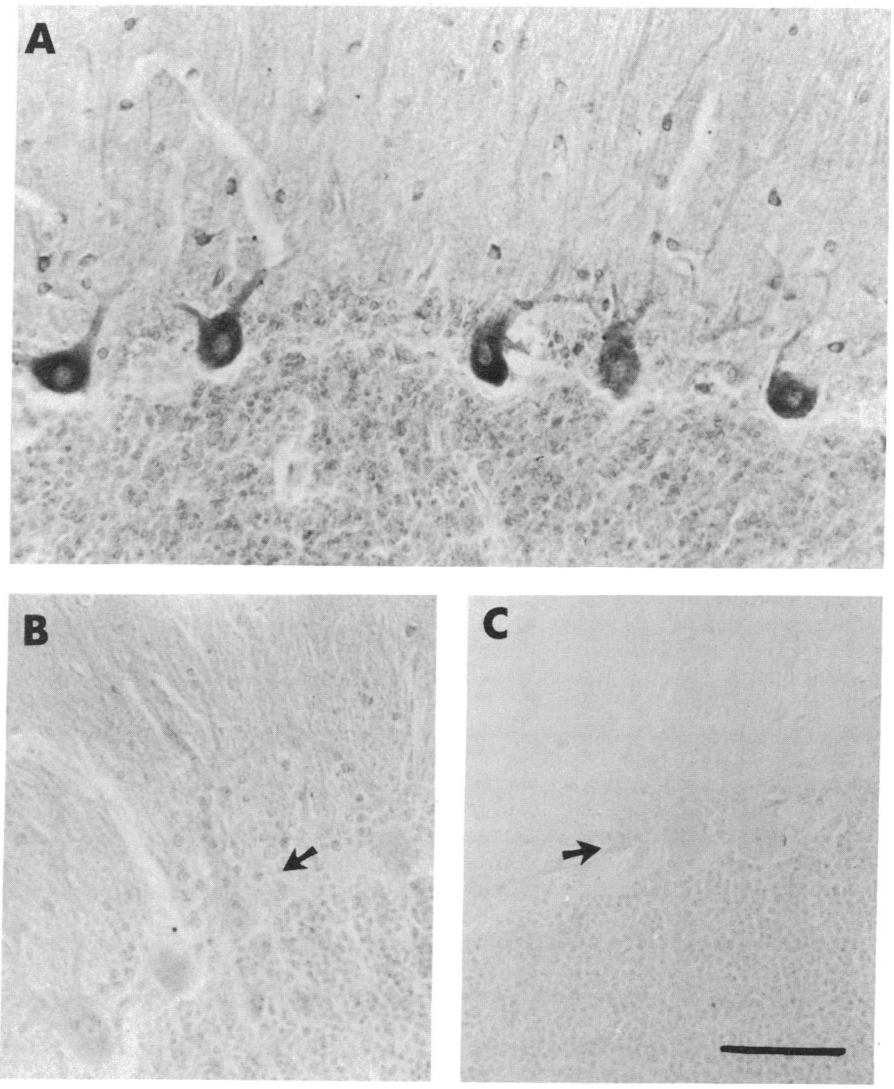

FIG. 6. Immunocytochemistry of human cerebellum stored in paraffin since 1940. (*A* and *B*) Reactivity with anti-benzodiazepine mAb 21-7F9 in the absence and presence of 0.1 μM diazepam, respectively. (*C*) Negative control using the same immunocytochemical procedure but with the culture medium from the parental myeloma line P3X63Ag8.6.5.3 in place of 21-7F9. Arrows show the Purkinje cell layer. (Bar = 100 μm.)

molecule containing a chlorine atom could be totally biosynthesized by mammals.

The immunocytochemical experiments with the human brain samples stored since 1940 indicate that the brain benzodiazepine-like molecules that are recognized by mAb 21-7F9 are of biological rather than chemical origin, since the first chemical synthesis of benzodiazepines was done in 1955 (22) and Librium (chlordiazepoxide) appeared in the market in 1960 (22). These immunocytochemical experiments support the existence of benzodiazepine-like molecules of natural origins. Nevertheless, we are not sure that the benzodiazepine-like immunoreactivities detected by immunocytochemistry and immunoblotting (1) in the brains correspond to the *N*-desmethyldiazepam that we isolated. Until this is established, we would like to leave open the possibility that the isolated benzodiazepine might have an industrial origin.

It is unlikely that *N*-desmethyldiazepam is a contaminant introduced during our purification procedure. (*i*) When the purification was done in control experiments in the absence of brain tissue, no benzodiazepine activity was recovered in any of the purification steps. (*ii*) Benzodiazepine-like immunoreactivity was detected not only in the biochemical experiments presented here but also by immunocytochemistry and immunoblotting. (*iii*) Contaminant benzodiazepines would have interfered with the receptor and antibody binding assays. (*iv*) *N*-Desmethyldiazepam or its precursors were seldom used in our laboratory at Stony Brook before the isolation of the compound from brain (the mass spectroscopy was done at the National Institutes of Health). We routinely use flunitrazepam and clonazepam in our benzodiazepine receptor binding assays, but these are not biotransformed into *N*-desmethyldiazepam (23).

A possibility worth considering is that *N*-desmethyldiazepam (a major metabolite of diazepam and of many other benzodiazepines) accumulates in the mammalian brain as a result of the intake of synthetic benzodiazepines that could be present in the drinking water or the animal food or even purposely given to cattle. This seems an unlikely possibility, especially in the case of laboratory rats that have been fed a controlled diet throughout their lives. If this were the real explanation for the presence of *N*-desmethyldiazepam in the brain, then serious toxicological implications would be derived from our observations. *N*-Desmethyldiazepam is a common metabolite that is formed in the body during the degradation of benzodiazepines such as diazepam, chlordiazepoxide, clorazepate, and prazepam (23). *N*-Desmethyldiazepam (nordiazepam) is also used as a prescription benzo-

diazepine. The elimination from the tissues of *N*-desmethyldiazepam is slow because of its low clearance rate. The elimination half-life of this substance in humans is between 50 and 100 hr (23). Furthermore, *N*-desmethyldiazepam and other benzodiazepines accumulate in the body after prolonged use. The slow elimination rate and the accumulation in the tissues seem to be due to the tight binding of these molecules to serum albumin and other tissue proteins (23). The protein immunoreactivities detected with the anti-benzodiazepine mAb 21-7F9 in immunoblotting experiments with rat brain (1) could result from benzodiazepines that are tightly bound to proteins and not from amino acid epitopes.

Regardless of the origin of the endogenous benzodiazepines, they seem to be mostly in bound form, attached to proteins and probably other biomolecules as well (although they are released during the purification procedure). These might represent not only storage compartments for their use as neuromodulators of γ-aminobutyric acid-mediated transmission but also compartments for their biotransformation, accumulation, and elimination. Benzodiazepines are lipophilic compounds that are converted to more hydrophilic metabolites and are conjugated to other biomolecules by the cytochrome P-450 enzyme system. This system plays a major role in the biotransformation of xenobiotics (drugs, mutagens, carcinogens, etc.) as well as endogenous substances such as steroids and prostaglandins (24).

Another possibility is that benzodiazepines could be biosynthesized by microorganisms and/or plants and that they become components of our diet (like vitamins, for example). These substances could not only affect γ-aminobutyric acid-mediated transmission but also accumulate in the brain tissue as discussed above. *N*-Desmethyldiazepam could be taken from the diet as such or in the form of a precursor that could be converted to *N*-desmethyldiazepam in the animal body. This interpretation is consistent with the results obtained with the human brains from 1940. [Some support for this hypothesis also comes from the demonstration of benzodiazepine biosynthesis in the fungus *Penicillium cyclopium* (25, 26).] If this were the case, it would be an example of functional modulation of a neurotransmitter (γ-aminobutyric acid) receptor by a dietary component (benzodiazepine), which in turn could affect brain function and behavior.

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