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Señor
SUPERINTENDENTE DE INDUSTRIA Y COMERCIO
DIVISION DE NUEVAS CREACIONES
E. S. D.

Re: P1.-F. HOFFMANN-LA ROCHE AG.-Solicitud de Patente de Invención en
Fase Nacional PCT para "DERIVADOS DE PIRIDO [2, 1-A]
ISOQUINOLINA COMO INHIBIDORES DE DPP-IV".-Clase C07D
471/04.-Expediente número 04-058.260.-

1. Me refiero al auto dictado por ese Despacho notificado mediante fijación en lista de 4 de Abril de 2008.
2. De acuerdo con el Concepto Técnico No. **854** se tiene que:

"Como consecuencia de la búsqueda de anterioridades de materia relacionada con la presente invención, se determinó que existen en el estado de la técnica los siguientes documentos que eventualmente afectarían los requisitos de patentabilidad de esta solicitud:

- **D.J. CANNEY ET AL.: "Amino- and Amido-Tetrabenazine Derivatives: Synthesis and Evaluation as Potential Ligands for the Vesicular Monoamine Transporter" NUCL. MED. BIOL., Vol. 22, no. 4, 1995, pages 527-535, XP002238994;**
- **CHEMICAL ABSTRACTS., vol. 108, no.17, 25 April 1988 (1988-04-25) Columbus, Ohio, US; Abstract no. 143909n, D. SCHERMAN ET AL.: "Hydrophobicity of the Tetrabenazine-Binding site of the Chromaffin Granule Monoamine Transporter", Page 124, XP002238995, abstract, & MOL. PHARMACOL., Vol. 33, no. 1, 1988, pages 72-77;**
- **CHEMICAL ABSTRACTS., vol. 103, no.5, 5 August 1985 (1985-08-05) Columbus, Ohio, US; Abstract no. 34404v, M.F. ISAMBERT, J. P. HENRY: "Photoaffinity Labeling of the Tetrabenazine Binding Sites of Bovine Chromaffin Granule Membranes" Page 243;**

XP002238996 abstract & BIOCHEMISTRY, Vol. 24, no. 14, 1985, pages 3660-3667;

- **CHEMICAL ABSTRACTS.**, vol. 78, no.7, 19 February 1973 (1973-02-19) Columbus, Ohio, US; abstract no. 43232e, A. BUZAS ET AL.: "Synthesis of 2-aminohexahydrobenzo [a] quinolizines" Page 466; XP002238997, Cited in the application abstract, & CHIM, THER., Vol. 7, no. 5, 1972, pages 404-407; and
- **CHEMICAL ABSTRACTS.**, vol. 54, no.13, 10 July 1960 (1960-07-10) Columbus, Ohio, US; abstract no. 13128a, A.R. BATTERSBY ET AL.: "Stereochemistry of emetine" XP002238998, abstract, & CHEM. & IND. (LONDON), 1957, page 982.

3. Para responder a los requerimientos del señor Examinador de conformidad con el Artículo 46 de la Decisión 486, anexo me permito presentar a ese Despacho copia de los siguientes documentos solicitados:

- * **D.J. CANNEY ET AL.:** "Amino- and Amido-Tetrabenazine Derivatives: Synthesis and Evaluation as Potential Ligands for the Vesicular Monoamine Transporter" NUCL. MED. BIOL., Vol. 22, no. 4, 1995, pages 527-535, XP002238994;
- * **CHEMICAL ABSTRACTS.**, vol. 108, no.17, 25 April 1988 (1988-04-25) Columbus, Ohio, US; Abstract no. 143909n, D. SCHERMAN ET AL.: "Hydrophobicity of the Tetrabenazine-Binding site of the Chromaffin Granule Monoamine Transporter", Page 124, XP002238995, abstract, & MOL. PHARMACOL., Vol. 33, no. 1, 1988, pages 72-77;
- * **CHEMICAL ABSTRACTS.**, vol. 103, no.5, 5 August 1985 (1985-08-05) Columbus, Ohio, US; Abstract no. 34404v, M.F. ISAMBERT, J. P. HENRY: "Photoaffinity Labeling of the Tetrabenazine Binding Sites of Bovine Chromaffin Granule Membranes" Page 243; XP002238996 abstract & BIOCHEMISTRY, Vol. 24, no. 14, 1985, pages 3660-3667;
- * **CHEMICAL ABSTRACTS.**, vol. 78, no.7, 19 February 1973 (1973-02-19) Columbus, Ohio, US; abstract no. 43232e, A. BUZAS ET AL.: "Synthesis of 2-aminohexahydrobenzo [a] quinolizines" Page 466; XP002238997, Cited in the application abstract, & CHIM, THER., Vol. 7, no. 5, 1972, pages 404-407; and
- * **CHEMICAL ABSTRACTS.**, vol. 54, no.13, 10 July 1960 (1960-07-10) Columbus, Ohio, US; abstract no. 13128a, A.R. BATTERSBY ET AL.: "Stereochemistry of emetine" XP002238998, abstract, & CHEM. & IND. (LONDON), 1957, page 982.



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Amino- and Amido-Tetrabenazine Derivatives: Synthesis and Evaluation as Potential Ligands for the Vesicular Monoamine Transporter

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Tetrabenazine (TBZ) and dihydrotetrabenazine are well known inhibitors of the CNS vesicular monoamine transporter (VMAT), which is responsible for the packaging of monoamine neurotransmitters in presynaptic vesicles. Amino and amido derivatives of tetrabenazine were prepared as potential ligands for the vesicular monoamine transporter. Ultimately, organotin derivatives of promising ligands were prepared for radiolabeling with ^{125}I . The compounds were evaluated for their ability to inhibit the specific binding of a selective radioligand to the transporter in rat striatal homogenates. Of the compounds evaluated, three amine derivatives of TBZ (primary, secondary and tertiary) were found to have modest to high affinity for the transporter, while two amides exhibited low to undetectable affinity. The secondary propargyl amine was found to possess the highest affinity ($K_i = 7.6 \text{ nM}$) and was chosen for further evaluation. The organotin derivative of this compound was synthesized in order to prepare the corresponding radioiodinated ligand. However, our inability to synthesize and characterize the iodinated amine precluded its evaluation as a potential radioiodinated ligand for the transporter. Alternative approaches for decreasing the lipophilicity of TBZ analogs while maintaining high binding affinity are currently being explored.

Introduction

Abnormalities in monoaminergic neurotransmission in the central nervous system (CNS) have been implicated in a number of psychological and neurological disorders (Cooper *et al.*, 1986). Therefore, CNS markers for the accurate determination of monoaminergic innervation are valuable tools for studying the possible role of catecholamine and indolamine neurotransmitter systems in these disorders. Synaptic neurotransmission in the CNS is closely coupled to the active transport and storage of dopamine, serotonin, and norepinephrine. Following the release of neurotransmitter into the synaptic cleft, two distinct active transport systems are involved in moving monoamines from the cleft to storage vesicles within the presynaptic neuron. First, termination of action often involves Na^+ -coupled reuptake (plasma membrane transport) of the transmitter to either the presynaptic nerve terminal or to glial cells (Kanner and Schuldiner, 1987; Schuldiner, 1994). Following uptake of the monoamines into the cytoplasm of the presynaptic neuron, a second type of transporter

(vesicular monoamine transporter, VMAT) is responsible for packaging and storage of the neurotransmitters in monoamine vesicles. The latter mechanism, VMAT, is based on common ATP-dependent transporters that are effective for both catecholamines and serotonin (Kanner and Schuldiner, 1987; Schuldiner, 1994). Reserpine is a natural product that inhibits the VMAT mechanism within the synapse, resulting in a depletion of monoamines in the CNS (Cooper *et al.*, 1986). The depletion of monoamines in the brain by reserpine is long-lasting and the storage mechanism appears to be irreversibly damaged. In contrast, the alkaloid tetrabenazine, [3-(2-methylpropyl)-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2H-benzo[a]-quinolizin-2-one; TBZ, **1** (Fig. 1)] produces a short lived depletion of monoamines, and does not appear to irreversibly damage the transporter (Cooper *et al.*, 1986; Neumeyer, 1981; Weiner, 1985).

A reduced form of TBZ, tritiated dihydrotetrabenazine [2-hydroxy-3-(2-methylpropyl)-9,10-dimethoxy-1,3,4,6,7,11b-hexahydro-2H-benzo[a]-quinolizine]; (^3H)DH-TBZ, **2**) has been reported as a selective marker for *in vitro* radioligand binding ($K_d = 2.9 \text{ nM}$) and autoradiographic studies of the VMAT system (Near, 1986; Darchen *et al.*, 1989;

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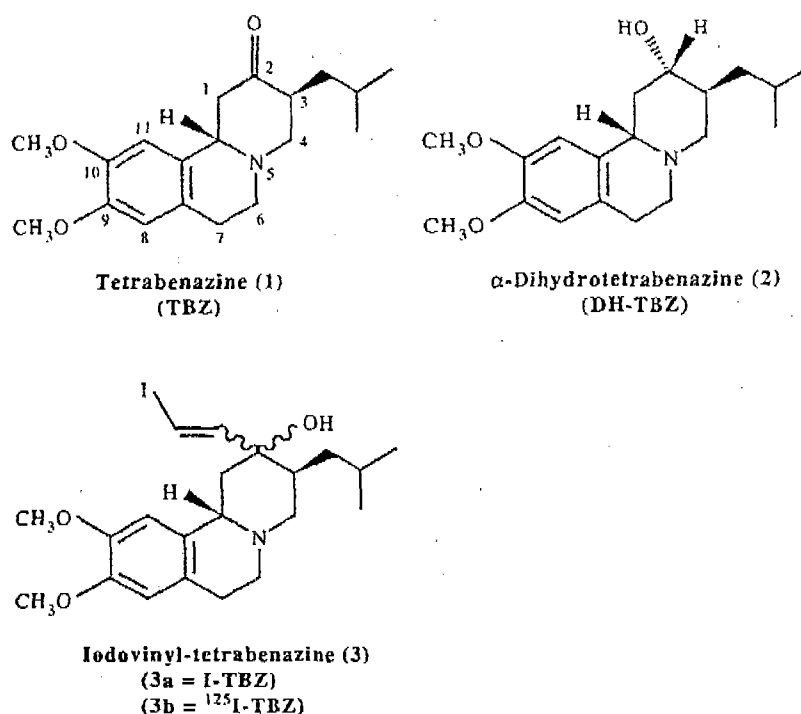


Fig. 1. Structures of tetrabenazine (TBZ; 1), dihydrotetrabenazine (DH-TBZ; 2), and iodovinyl-tetrabenazine (I-TBZ; 3) discussed in the text.

Henry and Scherman, 1989; Isambert *et al.*, 1989; Masuo *et al.*, 1990). Autoradiography studies have shown that [³H]DH-TBZ labels monoamine rich brain regions (Darchen *et al.*, 1989; Henry and Scherman, 1989; Masuo *et al.*, 1990). For example, postmortem binding studies using [³H]DH-TBZ demonstrated a significant reduction of [³H]DH-TBZ binding in the putamen and caudate nucleus of the brains of parkinsonian patients as compared to controls (Cooper *et al.*, 1986; Kanner and Schuldiner, 1987).

An ¹²⁵I ligand for the transporter site would significantly reduce the exposure times necessary for the tritiated ligand. When labeled with ¹²⁵I (specific activity 2000 Ci/mmol), a radioiodinated ligand specific for the transporter could be used for *in vitro* radioligand binding and autoradiographic studies. Additionally, an ¹²⁵I labeled ligand may be found useful for *in vivo* Single Photon Emission Computed Tomography (SPECT) studies. An iodinated ketanserin analog (known to possess moderate affinity to VMAT) has been synthesized, but has been shown to be less specific and to exhibit higher nonspecific binding than [³H]DH-TBZ (Darchen *et al.*, 1989; Henry and Scherman, 1989). Therefore, the development of specific, high affinity radioiodinated ligands for the vesicular monoamine uptake site remains an important objective.

Numerous factors must be taken into consideration when designing radioiodinated derivatives of TBZ as potential radioligands for *in vitro* (radioligand binding and autoradiography) and *in vivo*

(SPECT imaging) studies of the vesicular monoamine transporter. First, a previous study has demonstrated a correlation between the lipophilicity of TBZ analogs and affinity for the reuptake site (Scherman *et al.*, 1988b). Compounds shown to have higher partition coefficients (octanol/buffer) in the study generally exhibited a greater ability to inhibit the specific binding of [³H]DH-TBZ. While the correlation was based on a limited number of compounds, this observation is not surprising since the ability of these compounds to reach their intracellular targets would be expected to depend, at least in part, on their ability to pass through lipid bilayers of neuronal membranes. Second, structure-activity relationship (SAR) studies involving TBZ have shown that a basic amine nitrogen at position 5, methoxy groups at positions 9, 10, and an alkyl group at position 3 impart good amine depleting activity on the isoquinoline molecule (Brossi *et al.*, 1958; Pletscher *et al.*, 1962; Tretter, 1962; Fahrenholtz *et al.*, 1967; Harnden and Short, 1967; Lednicer and Mitscher, 1977; Saner and Pletscher, 1977; Kaiser and Setler, 1981). Additionally, SAR studies suggest that analogs containing considerable steric bulk at the 2 position retain amine depleting activity and/or high affinity for the uptake site (Scherman *et al.*, 1988a; Isambert *et al.*, 1989). These data suggest that derivatization at the 2 position of the TBZ molecule with substituents of the appropriate lipophilicity, could result in analogs that retain high affinity and specificity for the receptor site.

Our attempts to develop a radioiodinated TBZ analog for *in vivo* SPECT imaging studies of the

transporter have been disappointing thus far. A 2-iodovinyl TBZ (I-TBZ) ligand was shown to exhibit high specificity and affinity for VMAT *in vitro*. However, the high lipophilicity and consequent high nonspecific binding of I-TBZ have precluded its use as a radiotracer for SPECT imaging studies *in vivo* (Kung *et al.*, 1992a; Canney *et al.*, 1993). In an effort to reduce the lipophilicity of the iodovinyl ligand (I-TBZ), while maintaining high specificity and affinity for the transporter, amino and amido-TBZ analogs have been synthesized as potential precursors in the development of radioiodinated ligands for the vesicular monoamine transporter. Our goal was to develop ligands that would be sufficiently lipophilic to reach the intracellular target (vesicular monoamine transporter) and bind with high affinity and selectivity, while exhibiting a degree of hydrophilicity which might minimize nonspecific binding during *in vitro* binding assays, and during *in vivo* SPECT imaging studies. We herein report the synthesis of amino and amido-TBZ derivatives and the ability of these compounds to inhibit the specific binding of [¹²⁵I]-TBZ (Fraction I) in radioligand binding experiments.

Materials and Methods

General methods

Reagents used in syntheses were purchased from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.) or Fluka (Ronkonkoma, NY, U.S.A.) and used without further purification unless otherwise indicated. Anhydrous sodium sulfate (Na₂SO₄) was used as a drying agent. Reaction yields are reported without attempts at optimization. Reagent-grade and HPLC-grade solvents were obtained from Emsco Co. (Philadelphia, PA, U.S.A.). Toluene was refluxed over sodium metal and distilled prior to use. Dichloromethane was distilled from lithium aluminum hydride prior to use. Thin layer chromatography (TLC) was performed on EM Science (Gibbstown, NJ, U.S.A.) precoated (0.2 mm) silica gel 60 plates and the spots were detected with I₂ vapor and/or UV light. Silica gel 60 (70–230 mesh), obtained from EM Science (Gibbstown, NJ, U.S.A.) or silica gel, Merck, grade 60 (230–400 mesh), obtained from Aldrich Chemical Co. (Milwaukee, WI, U.S.A.), were used for column chromatography. ¹H spectra were obtained on a Bruker Model AM 500 or 300, or a Varian 360A spectrometer. All samples prepared for NMR analysis were dissolved in CDCl₃ purchased from Aldrich Chemical Co. Chemical shifts are reported as δ values with the chloroform or tetramethylsilane resonance used as the internal standard. The multiplicity is defined by *s* (singlet), *d* (doublet), *t* (triplet), *q* (quartet), and *m* (multiplet). The relative peak heights of the resonances are reported as integers after the multiplicity. IR spectra were recorded (KBr pellet or neat as a film) with a Mattson Polaris FT-IR spectrometer. Melting points were

determined on a Meltemp apparatus (Cambridge, MA, U.S.A.) and are reported uncorrected. Elemental analyses were performed by Atlantic Microlabs Inc. (Norcross, GA, U.S.A.). A Rainin liquid chromatograph equipped with a reverse phase cartridge (PRP-1; 25 cm × 0.46 cm; Hamilton) or a chiral column (Chiralcel OD; 25 cm × 0.46 cm; Daicel Chemical Industries, Ltd) was used for high-performance liquid chromatographic (HPLC) separations and analyses. No-carrier-added sodium [¹²⁵I]iodide (0.1 N NaOH solution; 2200 Ci/mmol) was purchased from DuPont NEN Research Products (Boston, MA, U.S.A.).

9,10-Dimethoxy-3-(2-methylpropyl)-1,3,4,6,7,11b-hexahydro-2H-benzo[a]quinolizin-2-amine (4a)

Compound 4a was prepared using a modification of a previously reported procedure (Isambert *et al.*, 1989). Tetrabenazine (2 g; 6.3 mmol), ammonium acetate (4.86 g; 63 mmol), and NaBH₃CN (0.28 g) were dissolved in anhydrous MeOH (50 mL). Molecular sieves (3 g; 3 Å) were added and the mixture was stirred at RT for 24 h. The solution was diluted with methanol (100 mL) and filtered. Methanol was removed *in vacuo* and the residue taken up in water (50 mL). The mixture was made acidic with concentrated HCl (pH < 4) and extracted with Et₂O (2 × 100 mL). The solution was then made basic with 5 N NaOH (pH > 10) and extracted with Et₂O (4 × 100 mL). The ether was washed with brine, dried (Na₂SO₄) and evaporated under reduced pressure. The residue recovered after work-up was chromatographed on a silica gel column and eluted with 12% MeOH-CHCl₃ to yield the known compound 4a (1.47 g; 73.5%) as a slightly yellow oil. Chromatographic properties and the spectroscopic data included below are consistent with reported values. ¹H NMR δ 6.69–6.68 (*m*, 1H), 6.56 (*s*, 1H), 3.83 (*s*, 6H), 3.12–3.02 (*m*, 3H), 2.89–2.80 (*m*, 1H), 2.72–2.18 (*m*, 6H), 2.04–1.77 (*m*, 1H), 1.75–1.36 (*m*, 4H), 1.12–0.98 (*m*, 1H) 0.95–0.87 (*m*, 6H); IR (film) 3380, 2960, 2920, 2840/2760 *m* (Bohlmann bands), 1640, 1520, 1460, 1370, 1330, 1260, 1240 cm⁻¹.

9,10-Dimethoxy-3-(2-methylpropyl)-N-2-propynyl-1,3,4,6,7,11b-hexahydro-2H-benzo[a]quinolizin-2-amine (4b)

Compound 4b was prepared using the reductive amination procedure of Borch *et al.* (1971). To a solution of propargylamine (7.78 mL; 113 mmol) in anhydrous MeOH (40 mL) was added 5 N HCl-MeOH (7.56 mL; 38 mmol). Tetrabenazine (6 g; 19 mmol) was then added, followed by NaBH₃CN (0.83 g). Molecular sieves (10 g; 3 Å) were added and the mixture was stirred at RT for 72 h. The solution was diluted with methanol (150 mL) and filtered. Methanol was removed *in vacuo* and the residue taken up in water (30 mL). The mixture was made acidic with concentrated HCl (pH < 4) and extracted

and moderate lipophilicity be identified. The optimal ligand would be sufficiently lipophilic to reach the target receptor, and hydrophilic enough so that non-specific binding is minimized during *in vitro* binding experiments, and perhaps more importantly, during *in vivo* SPECT imaging studies. While a highly lipophilic radioiodinated ligand has been shown to be useful (I-TBZ; 3b) for *in vitro* binding experiments (Kung, 1994), high nonspecific binding *in vivo* precluded its use in SPECT imaging studies.

In conclusion, 2-amino and 2-amido derivatives of TBZ were prepared in low to moderate yield. The compounds were evaluated for their ability to inhibit the binding of specifically bound 3b (Fraction I) from rat striatal membranes. Of the compounds evaluated here, propargylamine 4b exhibited the highest affinity for the transporter and possessed a triple bond which could readily be further functionalized to obtain the organotin precursor and ultimately the target iodinated compound. However, an inability to prepare 8 precluded its evaluation as a potential radioiodinated ligand for the transporter. We are currently exploring alternative approaches to decrease the lipophilicity of this series of TBZ analogs while maintaining the desired high binding affinity.

Radiolabeling

The I-125 labeled iodovinyl-TBZ derivative ([¹²⁵I]-TBZ, 3b) was prepared as previously described by an iododestannylation reaction (Canney *et al.*, 1993). The product was purified by HPLC on a reverse phase column (PRP-1, Hamilton) eluting with an isocratic solvent of 90% acetonitrile–10% buffer (5 mM 3,3'-dimethylglutaric acid, pH 7.0) and flow rate of 1 mL/min. The radiochemical purity was analyzed by the above chromatographic system. The specific activity of 3b was not determined, however, based on our experience with similar iododestannylation reactions where the specific activity was determined to be approximately 2200 Ci/mmol, we feel that it is reasonable to assume that the specific activity of no carrier-added 3b is also 2200 Ci/mmol.

Separation of optical isomers was achieved using a Rainin HPLC system in conjunction with a chiral column (Chiracel OD) as described previously (Canney *et al.*, 1993). The overall yield obtained for 3b following reverse-phase and Chiral HPLC separations was 50%. *K_i* values reported herein were determined in binding experiments using 3b, Fraction I as the radioligand (*K_d* 0.3 nM) (Kung *et al.*, 1992a).

Partition Coefficients

The following procedure was used to determine partition coefficients. The radioligand was mixed with 3 g each of 1-octanol and buffer (pH 7.4, 0.1 M phosphate) in a test tube. The mixture was vortexed for 3 min at RT and then centrifuged (4000 rpm) for 5 min. Two aliquots from each layer (0.5 g each) were

counted in a well counter. The partition coefficients were determined by calculating the ratio of cpm/g of octanol to that of buffer. Samples from the octanol layer were repartitioned until consistent values were obtained (usually after 3 determinations).

Binding Assay

Rat striatal homogenates were prepared following the procedure described (Scherman *et al.*, 1986). The binding was carried out in a total volume of 0.2 ml containing buffer (50 mM HEPES, pH 7.5, 0.3 M sucrose), [¹²⁵I]-labeled ligand, tissue homogenates (100–150 µg) and various concentrations of competitors. Incubation was carried out for 60 min at RT. The samples were then filtered through glass fiber filters (Schleicher and Schuell, No. 30) and the bound radioactivity was determined in a γ counter (Packard 5000). The non-specific binding was measured in the presence of 1 µM tetrabenzine. The data points were analyzed using the iterative, nonlinear least square curve fitting program LIGAND (Munson and Robard, 1980).

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Appendix 1. Analytical data

Compound	Calculated			Found		
	C	H	N	C	H	N
4b	74.12	9.05	7.86	74.20	9.01	7.92
4c	74.56	9.25	7.56	74.27	9.26	7.44
5a	69.97	8.95	7.77	70.01	9.00	7.69
5b	56.94	6.06	5.11	56.84	6.05	5.08
6	64.14	8.50	3.94	64.06	8.48	3.98
7	63.06	9.34	4.33	63.14	9.30	4.28

108:143909n Hydrophobicity of the tetrabenazine-binding site of the chromaffin granule monoamine transporter. Scherman, Daniel; Gasnier, Bruno; Jaudon, Pascale; Henry, Jean Pierre (Inst. Biol. Phys. Chim., 75005 Paris, Fr.). *Mol. Pharmacol.* 1988, 33(1), 72-7 (Eng). The catecholamine uptake inhibitor tetrabenazine (TBZ) binds to a high affinity site on the chromaffin granule membrane, presumably on the monoamine transporter. The hydrophobicity of the TBZ-binding site was investigated by comparing the potency of drugs to displace [^{3} H]dihydrotetrabenazine (TBZOH), a ligand of the TBZ-binding site, with the lipophilicity of these drugs reflected by their octanol/buffer apparent partition coeff. ($P_{o/b}$). Drugs tested were 5 substrates of the transporter, 7 TBZ derivs., and the inhibitors reserpine, haloperidol, and chlorpromazine. The validity of apparent $P_{o/b}$ as an index of lipophilicity was shown by measuring drug partitioning between buffer and chromaffin granule membranes. For most of the inhibitors tested, octanol/buffer and membrane/buffer apparent $P_{o/b}$ were correlated. For substrates of uptake and TBZ derivs., the potency of a compd. to displace [^{3} H]TBZOH from its binding site was correlated to its apparent $P_{o/b}$. This relation was valid over a range of 5 orders of magnitude. These data are interpreted as indicating that the TBZ-binding site is hydrophobic and is in equil. with the ligand present in the membrane phase, and that substrates and TBZ derivs. are characterized by an equal intrinsic affinity for this site of $\sim 1 \mu\text{M}$. The 3-fold difference in affinity obsd. between α - and β -diastereoisomers of TBZOH was accounted for by a similar difference in apparent $P_{o/b}$. Reserpine, haloperidol, and chlorpromazine have much lower intrinsic affinity for the TBZ-binding site.

Hydrophobicity of the Tetrabenazine-Binding Site of the Chromaffin Granule Monoamine Transporter

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SUMMARY

The catecholamine uptake inhibitor tetrabenazine (TBZ) binds to a high affinity site on the chromaffin granule membrane, presumably on the monoamine transporter. The hydrophobicity of the TBZ-binding site was investigated by comparing the potency of drugs to displace [^{3} H]dihydrotetrabenazine (TBZOH), a ligand of the TBZ-binding site, with the lipophilicity of these drugs reflected by their octanol/buffer apparent partition coefficient ($P_{o/b}$). Drugs tested were five substrates of the transporter, seven TBZ derivatives, and the inhibitors reserpine, haloperidol, and chlorpromazine. The validity of apparent $P_{o/b}$ as an index of lipophilicity was shown by measuring drug partitioning between buffer and chromaffin granule membranes. For most of the inhibitors tested, octanol/buffer and membrane/buffer apparent partition coefficients were correlated.

For substrates of uptake and TBZ derivatives, the potency of a compound to displace [^{3} H]TBZOH from its binding site was correlated to its apparent $P_{o/b}$. This relationship was valid over a range of 5 orders of magnitude. These data are interpreted as indicating that the TBZ-binding site is hydrophobic and is in equilibrium with the ligand present in the membrane phase, and that substrates and TBZ derivatives are characterized by an equal intrinsic affinity for this site of about 1 μ M. The 3-fold difference in affinity observed between α - and β -diastereoisomers of TBZOH was accounted for by a similar difference in apparent $P_{o/b}$. Reserpine, haloperidol, and chlorpromazine have much lower intrinsic affinity for the TBZ-binding site.

Chromaffin granules, the catecholamine storage organelles of adrenal medulla, accumulate monoamines (catecholamines and indoleamines) against their concentration gradient (1, 2). This active transport is mediated by a specific transporter driven by the proton electrochemical gradient generated by an ATP-dependent proton pump (3-8). Two specific inhibitors of this transport are known, reserpine and TBZ, which inhibit the monoamine transporter without affecting the electrochemical H^+ -gradient (9, 10). Using [^{3} H]reserpine and a tritiated analog of TBZ, [^{3} H]TBZOH, inhibition of uptake has been shown to result from the binding of these drugs to high affinity sites on the monoamine transporter (11-13).

The TBZ-binding site (site T) has a low affinity for the substrates of uptake, catecholamines and indoleamines, as measured by displacement of bound [^{3} H]TBZOH. For noradrenaline, the dissociation constant derived from these measurements is in the mM concentration range and is 2 orders of magnitude larger than its K_M for ATP-dependent noradrenaline

uptake (11). This difference is surprising, in view of the structural analogy existing between TBZ and catecholamines (Fig. 1). We thus investigated the possibility that the observed differences in the binding constants for site T of TBZ and of substrates might originate in differences in their physicochemical properties. In the present communication, this hypothesis was tested by comparing the potency of substrates and inhibitors of the transporter to displace [^{3} H]TBZOH with their hydrophobicity, determined from partition experiments with the octanol/buffer model system.

Materials and Methods

Chemicals. TBZ (Fluka) and reserpine (Sigma) stock solutions (1 mM) were prepared in 2 mM HCl and 200 mM acetic acid, respectively, and were kept at 4° in the dark. MIBG and [^{125} I]MIBG were gifts from Office des Rayonnements Ionisants, Commissariat à l'Energie Atomique (Gif sur Yvette, France). α - and β -[^{3} H]TBZOH (15 Ci/mmol) were synthesized either in the laboratory or by the Commissariat à l'Energie Atomique. Drugs were generally solubilized in 2 mM HCl, at 1 mM final concentration.

Synthesis of TBZ derivatives. [^{3} H]TBZOH, [2- ^{3} H] 2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)-benzo[a]quino-

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ABBREVIATIONS: TBZ, tetrabenazine; TBZOH, dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)-benzo[a]quinolizine); [^{3} H]TBZOH, [2- ^{3} H]dihydrotetrabenazine; TBZNH₂, 2-aminotetrabenazine; TBA, arylazido tetrabenazine (*N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)-benzo[a]quinolizine-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide); 5-HT, 5-hydroxytryptamine (serotonin); MIBG, meta-iodobenzylguanidine; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; apparent $P_{o/b}$, octanol/buffer apparent partition coefficient; apparent $P_{m/b}$, membrane/buffer apparent partition coefficient.

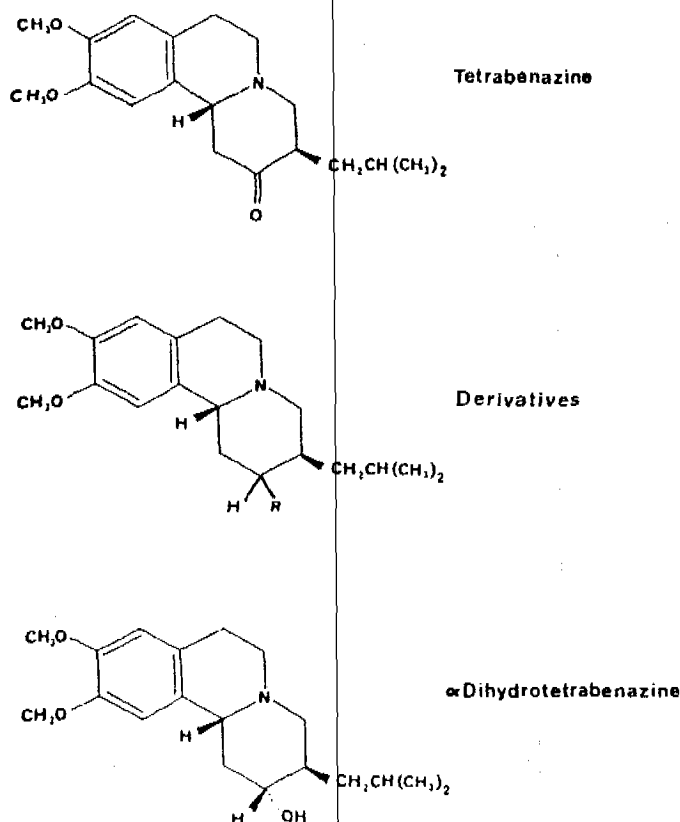


Fig. 1. Structural formulas of TBZ and TBZ derivatives. β -TBZOH is the diastereoisomer of α -TBZOH, which differs by an inverse configuration of the chiral carbon bearing the OH group. For the studied TBZ derivatives: acetyl TBZOH, $R = \text{OCOCH}_3$; succinyl TBZOH, $R = \text{OCO}(\text{CH}_2)_2\text{COOH}$; TBZNH₂, $R = \text{NH}_2$; TBA, $R = \text{NHCO}(\text{CH}_2)_3\text{NHC}_6\text{H}_3(\text{NO}_2)_3$.

lazine (dihydrotetrabenazine), was prepared by reduction of TBZ with KB^9H_4 (11). α and β OH isomers were purified and separated by high performance liquid chromatography on a C_{18} μ Bondapak column with methanol/10 mM $(\text{NH}_4)\text{CO}_3\text{H}$ (70:30) as solvent. Unless indicated, [^3H] TBZOH indicates the more abundant α isomer. The purity of this compound was periodically checked and, if necessary, it was repurified by high performance liquid chromatography. TBZNH₂ (2-amino TBZ), TBA (*N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b(H)-benzo[*a*]quinolizine-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide), an arylazido derivative of TBZNH₂, and [^3H]TBA were prepared as described (14). Succinyl and acetyl TBZOH were prepared by reaction of unfractionated TBZOH isomers with succinic and acetic anhydrides, respectively. Acetic or succinic anhydride (30 mmol) was added to TBZOH (1 mmol) in 2 ml of dry pyridine. After one night at room temperature, the solvent was removed. The product was dissolved in water (10 ml) and sodium carbonate was added to neutrality. The aqueous phase was extracted three times with CH_2Cl_2 (10 ml) and the combined organic phases were evaporated. Succinyl or acetyl TBZOH esters were crystallized from ether.

Chromaffin granule membrane preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer (15, 16). The preparation was frozen in liquid nitrogen and was kept at -80° . Protein concentration was estimated by the Lowry procedure (17).

Drug concentration determination. Concentrations of free drug (neutral and protonated) were determined spectrophotometrically for concentrations higher than $10\ \mu\text{M}$ and, for lower concentrations, fluorometrically or by radioactivity measurements. Spectrophotometry and fluorescence measurements were performed in 1 mM HCl because the

fluorescence of the protonated form of TBZ derivatives is more intense than that of the neutral form and because protonation is complete under these conditions. Absorbance and fluorescence characteristics were: TBZ derivatives, $\epsilon_{284} = 3,600\ \text{M}^{-1} \times \text{cm}^{-1}$, fluorescence/excitation 285 nm/emission 315 nm; reserpine, $\epsilon_{287} = 17,000\ \text{M}^{-1} \times \text{cm}^{-1}$ and fluorescence/excitation 295 nm/emission 365 nm; haloperidol, $\epsilon_{247} = 11,900\ \text{M}^{-1} \times \text{cm}^{-1}$; chlorpromazine, $\epsilon_{254} = 40,000\ \text{M}^{-1} \times \text{cm}^{-1}$; noradrenaline, adrenaline, serotonin, and tyramine, fluorescence/excitation 280 nm/emission 320 nm and $\epsilon_{275} = 15,000\ \text{M}^{-1} \times \text{cm}^{-1}$ for 5-HT. For TBZ derivatives, a common molecular absorption coefficient was assumed, which was verified by weighing in the case of TBZOH and succinyl TBZOH. For noradrenaline and adrenaline, fluorescence was also measured after adrenochrome formation (18).

[^3H]TBZOH binding. Membranes (10 μg of protein/ml) in 0.3 M sucrose/50 mM Hepes (Na) buffer, pH 7.5, containing 2.5 mM ATP/1.3 mM MgSO_4 , were incubated for 2 hr at 30° with 2 nM [^3H]TBZOH and the studied compound at various concentrations. Bound [^3H] TBZOH was determined by filtration through Millipore HAWP filters (11). Nonspecific binding, determined by addition of 1 μM TBZ to the incubation mixture, was subtracted. The drug concentration corresponding to half-inhibition of [^3H]TBZOH binding (EC_{50}) was derived from the inhibition curve.

Octanol/buffer apparent partition coefficient (apparent $P_{o/b}$). Apparent $P_{o/b}$ determinations were performed essentially as in Ref. 19. Briefly, compounds were added to 20 mM Hepes (Na) (pH 7.5) saturated with octanol. Water-saturated octanol was then added, the mixture was stirred for 5 min at 20° and centrifuged for 15 min at $7500 \times g$ at the same temperature, and the two phases were separated. For lipophilic inhibitors, which have an apparent $P_{o/b} > 1$, their concentration in the aqueous phase was measured as described above, and the concentration in octanol was calculated as the difference between total and aqueous phase concentrations. For hydrophilic uptake substrates with apparent $P_{o/b} < 1$, the octanol phase content was determined by reextracting this phase with an equal volume of 1 mM HCl. The concentration in the initial aqueous phase was obtained by subtraction.

Drug concentrations and volumes of the aqueous buffer and octanol phases were adjusted to give final concentrations in the 1–10 μM range in the aqueous phase for hydrophobic compounds or in the organic phase for hydrophilic compounds. Usually, five different concentrations were used. At the total concentration used for hydrophobic compounds (10–5000 μM), nonspecific adsorption on plastic tube walls could be neglected; however, these compounds were more strongly adsorbed to the plastic cones used to pipette the aqueous phase at pH 7.5, and the cones were thus routinely washed with 1 mM HCl.

Chromaffin granule membrane/buffer apparent partition coefficient (apparent $P_{m/b}$). Nonspecific adsorption of drugs to chromaffin granule membranes was determined in 20 mM Hepes (Na) buffer (pH 7.5) at 20° . Drugs were added to 50–200 μl of membranes in a Beckman Ultraclear Airfuge tube. After stirring for 1 min, the mixture was centrifuged for 20 min at $180,000 \times g$ and the free drug in the supernatant was assayed as described above. Drug adsorbed to the membranes was obtained as the difference between total and free drug. Each experiment was performed as rapidly as possible to minimize time-dependent adsorption of the ligand onto centrifuge tube walls.

Membrane concentration was adjusted in such a way that 20–80% of the drug was adsorbed to the membrane. For each compound, the experiment was performed at several drug concentrations, and it was verified that the apparent partition coefficient was independent of membrane and drug concentrations. With the exception of TBA, the concentrations used were 3 orders of magnitude larger than the K_D for specific binding of the drug to the monoamine transporter. Specific binding could thus be neglected under these conditions. For [^3H]TBA at low concentrations, the monoamine transporter was saturated by 5 μM TBZ.

The membrane/buffer apparent partition coefficient, $P_{m/b}$, expressed in ml/g of membrane protein, was derived from the experimental data

according to the equation, $P_{m/b} = [\text{Adsorbed drug}]/[\text{Free drug}] \times [\text{Membrane concentration}]$.

The apparent concentration ratio between the buffer phase and the hydrophobic phospholipid bilayer phase was tentatively deduced from $P_{m/b}$ using a value of 1.30 ml/g of membrane protein for the volume of the phospholipid bilayer in chromaffin granule membranes. This value was calculated using figures of 2.15 mmol/g of membrane protein for the phospholipid content of chromaffin granule membrane¹ (20), 2 nm for the mean length of a phospholipid acyl chain, and 2×10^6 phospholipid molecules/ μm^2 for the phospholipid surface density in a bilayer.

Results

Effect of drugs on [³H]TBZOH binding. We investigated the effects of 15 different compounds (see formulas in Fig. 1) on [³H]TBZOH binding to chromaffin granule membranes. [³H]TBZOH displacement curves are shown in Fig. 2. Compounds 1–4 are classical monoamine substrates of the transporter (5-HT, noradrenaline, adrenaline, and tyramine), which have a K_M in the μM concentration range; compound 6, MIBG, is an adrenal medulla imaging agent, which has recently been shown to be a substrate of the monoamine transporter ($K_M = 10 \mu\text{M}$) and to have a higher affinity for site T than endogenous substrates (21). Compound 5 is reserpine, which inhibits ATP-dependent monoamine uptake in the sub-nM concentration range. Compounds 7–13 are various derivatives of TBZ. Compounds 14 and 15 are, respectively, haloperidol and chlorpromazine, which inhibit the transporter in the μM concentration range. A Hill plot of the data of Fig. 2 indicated Hill coefficients ranging from 0.82 to 1.19. It was thus assumed that EC_{50} values derived from this experiment and indicated in Table 1 reflected a simple competition process, following a Michaelis-Menten model. The results of Table 1 indicate a large range of affinity for site T, EC_{50} values differing by more than 5 orders of magnitude from TBZ to substrates.

It may be noted that site T has some stereospecificity since the two diastereoisomers obtained by reduction of the keto group of TBZ, α - and β -TBZOH, have a different affinity for the monoamine transporter (Fig. 3). α -TBZOH has a 3- to 4-fold higher affinity for site T than β -TBZOH (Table 1, Fig. 3).

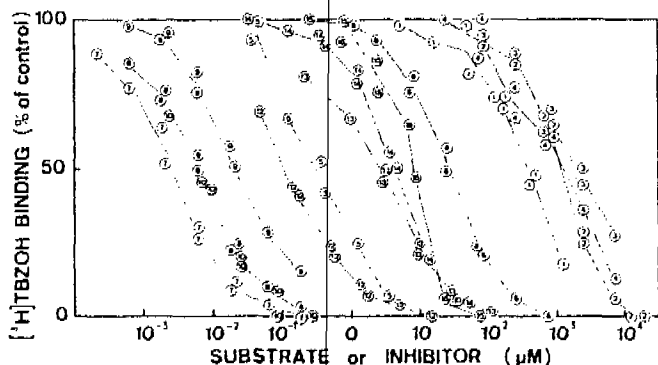


Fig. 2. Displacement of [³H]TBZOH by various substrates and inhibitors of the monoamine transporter. The numbers refer to compounds listed in Table 1. The Hill coefficients and the linear regression coefficients derived from a Hill plot of the data were: 1, -1.19 ($r = 0.99$, $n = 6$); 2, -1.10 ($r = 0.97$, $n = 8$); 3, -0.87 ($r = 0.97$, $n = 6$); 4, -0.82 ($r = 0.96$, $n = 6$); 5, -1.03 ($r = 0.99$, $n = 6$); 6, -1.07 ($r = 0.99$, $n = 7$); 7, -0.94 ($r = 0.99$, $n = 8$); 8, -0.90 ($r = 0.99$, $n = 8$); 9, -1.00 ($r = 0.99$, $n = 7$); 10, -0.93 ($r = 0.99$, $n = 5$); 12, -0.92 ($r = 0.99$, $n = 7$); 13, -0.87 ($r = 0.99$, $n = 8$); 14, -1.12 ($r = 0.99$, $n = 7$); 15, -1.19 ($r = 0.91$, $n = 6$).

¹ D. Scherman and J. P. Henry, unpublished data.

TABLE 1

Effect of substrates and inhibitors on [³H]TBZOH binding

Compound		Type ^a	[³ H]TBZOH binding, EC_{50} μM
No.	Name		
1	5-HT	S	330
2	Noradrenaline	S	1000
3	Adrenaline	S	1000
4	Tyramine	S	1000
5	Reserpine	I	0.3
6	MIBG	S	26
7	TBZ	I	0.003
8	α -TBZOH	I	0.006
9	β -TBZOH	I	0.020
10	Acetyl TBZOH	I	0.006
11	TBA	I	0.047 ^b
12	TBZNH ₂	I	0.110
13	Succinyl TBZOH	I	2.5
14	Haloperidol	I	4.0
15	Chlorpromazine	I	8.0

^a S, substrate; I, inhibitor.

^b Taken from Ref. 14.

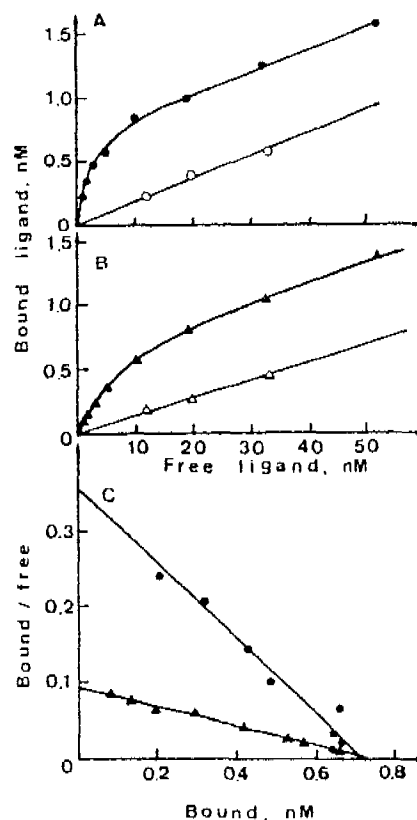


Fig. 3. α - and β -[³H]TBZOH binding isotherms. Membranes (12 μg of protein/ml) were incubated for 2 hr at 25° in 0.3 M sucrose/10 mM Hepes (Na), pH 8.0, with various concentrations of α - and β -[³H]TBZOH. A. α -[³H]TBZOH binding in the absence (●) or presence (○) of 1 μM TBZ. B. β -[³H]TBZOH binding in the absence (▲) or presence (△) of 1 μM TBZ. C. Scatchard plots of α -[³H]TBZOH (●) and β -[³H]TBZOH (▲)-specific binding. Characteristics of the binding were: for α -TBZOH (●), $K_D = 2.0$ nM and $B_{\text{max}} = 59.5$ pmol/mg of protein ($r = 0.967$); for β -[³H]TBZOH (▲), $K_D = 7.9$ nM and $B_{\text{max}} = 60.5$ pmol/mg of protein ($r = 0.982$).

162
162
75

Octanol/buffer and membrane/buffer apparent partition coefficients. The octanol/buffer partition coefficient of the investigated substrates and inhibitors of the monoamine transporter was determined. Since the partition coefficient of the protonated and the nonprotonated forms of these basic compounds are different (19), partition experiments were performed at pH 7.5, the pH value chosen for [³H]TBZOH displacement experiments, and the results were expressed as an apparent partition coefficient at that pH value, $P_{o/b}$, describing the partition of the studied molecule, both protonated and unprotonated (Table 2). Uptake inhibitors concentrated in the organic phase, thus indicating a marked hydrophobicity. For these compounds, nonspecific adsorption to the membrane, observed under conditions where specific TBZ-binding sites on the transporter were saturated, was also measured. It was expressed as membrane/buffer apparent partition coefficient, $P_{m/b}$ (Table 2).

For reserpine and most of the TBZ derivatives, apparent $P_{o/b}$ and $P_{m/b}$ were correlated ($p \leq 0.01$; $r = 0.974$, $n = 5$). The slope of the regression line ($\pm$ SE) is 0.59 ± 0.08 (Fig. 4). However, when data for haloperidol, chlorpromazine, and TBA were included, no correlation was observed ($p > 0.25$); for these three compounds, the observed apparent $P_{m/b}$ is larger than 0.6 times $P_{o/b}$. This point will be discussed below.

Relationship between the apparent affinity of the ligands for site T and their octanol/buffer apparent partition coefficient. For TBZ derivatives and substrates of uptake, there was an excellent correlation between the EC_{50} for [³H]TBZOH displacement and the apparent $P_{o/b}$. On a log-log plot (Fig. 5), the regression line corresponding to these compounds (Fig. 5, solid circles) had a slope ($\pm$ SE) of -1.069 ± 0.083 ($r = 0.971$; $p \leq 0.0001$). This plot indicated a linear reciprocal relationship between EC_{50} and apparent $P_{o/b}$. From the y axis intercept (0.675 ± 0.169 , SE), the following relation was deduced:

$$EC_{50} \times \text{apparent } P_{o/b} = 4.7 \mu\text{M} \quad (1)$$

TABLE 2
Octanol/Buffer and membrane/buffer apparent partition coefficients
Results are the mean of 5–10 determinations.

No.	Compound Name	Octanol/buffer apparent partition coefficient ($P_{o/b}$)	Membrane/buffer apparent partition coefficient ($P_{m/b}$)
			ml/g of protein
1	5-HT	0.0135	
2	Noradrenaline	0.0050	
3	Adrenaline	0.0028	
4	Tyramine	0.048	
5	Reserpine	5,200	3,270
6	MIBG	1.5	
7	TBZ	480	403
-	TBZOH*	105	130
8	α -TBZOH	189	
9	β -TBZOH	62	
10	Acetyl TBZOH	1,900	612
11	TBA	103	6,120
12	TBZNH ₂	34	217
13	Succinyl TBZOH	0.6	
14	Haloperidol	534	2,300
15	Chlorpromazine	1,315	10,950

* -, unfractionated mixture of α and β isomers (70:30).

Hydrophobicity of Tetrabenazine-Binding Site

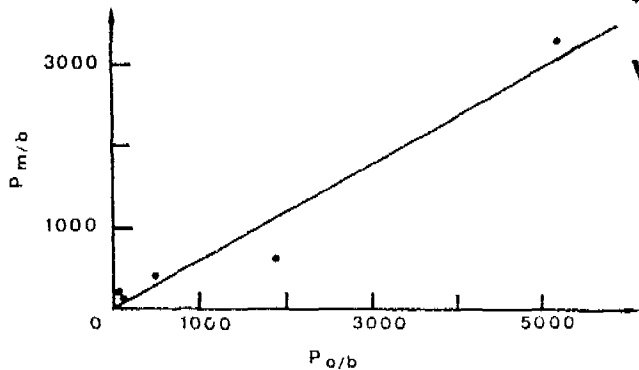


Fig. 4. Correlation between octanol/buffer ($P_{o/b}$) and membrane/buffer ($P_{m/b}$) apparent partition coefficients. Data for TBZ, TBZOH, acetyl TBZOH, TBZNH₂, and reserpine obtained from Table 2 are plotted. The line was fitted to the points by a least squares analysis ($r = 0.974$, $p \leq 0.01$).

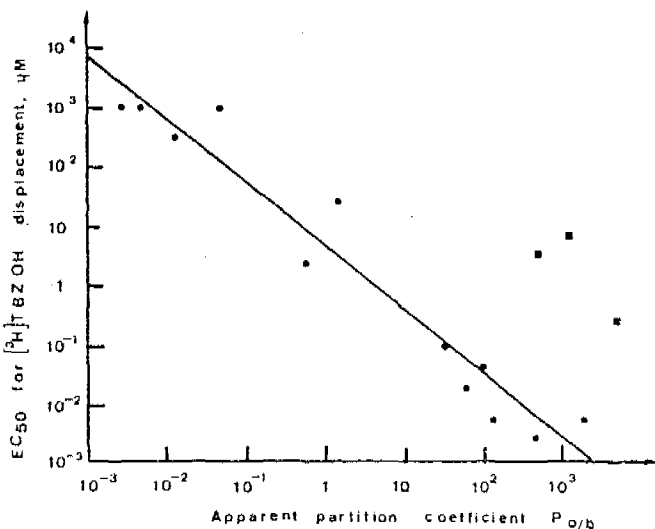


Fig. 5. Correlation between octanol/buffer ($P_{o/b}$) apparent partition coefficient and EC_{50} for [³H]TBZOH displacement. Data were taken from Tables 1 and 2. ●, substrates of uptake and TBZ derivatives; ■, reserpine, haloperidol, and chlorpromazine. Characteristics of the regression line derived from data on substrates of uptake and TBZ derivatives (●) were: $r = 0.971$, $p \leq 0.0001$; slope ($\pm$ SE): -1.069 ± 0.083 ; y axis intercept: 0.675 ± 0.169 ($\pm$ SE).

This relationship is valid for the various TBZ derivatives tested, the apparent $P_{o/b}$ of which extends over a range of 3 orders of magnitude. Moreover, the relationship is also valid for the substrates of the transporter, which are less hydrophobic than TBZ derivatives, thus extending the validity domain of the correlation to 5 orders of magnitude in apparent $P_{o/b}$. Among the substrates, tyramine and MIBG are located above the line. For these two compounds, EC_{50} values are about 10 times larger than those calculated from relation 1 (Eq. 1).

The regression line thus obtained does not fit the points corresponding to reserpine, chlorpromazine, and haloperidol, which are indicated by the solid squares in Fig. 5. The affinity of these compounds for TBZ-binding sites cannot be described by Eq. 1, which would underestimate their EC_{50} by 3 orders of magnitude.

Discussion

Octanol/buffer and membrane/buffer apparent partition coefficients. The octanol/buffer system has been pro-

posed as a model to study the apparent partition of hydrophobic compounds between aqueous buffers and biological membranes (22). The apparent $P_{o/b}$ is generally proportional to the apparent $P_{m/b}$ derived from nonspecific adsorption to membranes (22). We have observed such a linear relationship for reserpine and for most of the TBZ derivatives (Fig. 4). For TBA, haloperidol, and chlorpromazine, apparent membrane partitioning is larger than predicted from apparent $P_{o/b}$. A similar behavior of chlorpromazine has been reported in the study of the erythrocyte membrane (22). This behavior of chlorpromazine might be attributed to ionic interactions between the drug and membrane proteins, since apparent $P_{m/b}$ decreases with increasing ionic strength (23). Such an interpretation might also be proposed for TBA, which bears three amine functions. Thus, apparent $P_{o/b}$ which, contrary to apparent $P_{m/b}$, is not affected by these ionic interactions, seems to reflect better the liposolubility of a compound and, hence, the concentration ratio between the aqueous buffer and the membrane phase.

It must be noted that, for chlorpromazine, the apparent $P_{m/b}$ in chromaffin granule membranes is similar to that reported in erythrocyte membranes, when measured under the same low ionic strength conditions and expressed in the same units. A value of 3200 ml/g dry weight has been reported for erythrocyte membranes (23) and a value of 4200 ml/g dry weight was calculated for chromaffin granule membranes, assuming for the latter a figure of 3.4 g of membrane dry weight/g of protein (20).

Another advantage of the octanol/buffer system is that it can be used to estimate the liposolubility of the hydrophilic monoamine substrates. To derive an apparent $P_{m/b}$ for these compounds from nonspecific adsorption to membranes would be difficult, since substrates are transported and trapped by closed structures. Adrenaline, noradrenaline, and 5-HT have a low lipophilicity (Table 2). Tyramine and MIBG are more lipophilic, thus accounting for the lower plateau value of uptake observed with these two compounds (10, 21): influx rates are comparable to that of catecholamines and 5-HT, but efflux rates are higher for the lipophilic compounds, due to diffusion through the lipid bilayer.

Intrinsic affinity of substrates and inhibitors for TBZ-binding site. The major finding of this work is the large validity domain of the linear correlation existing between the reciprocal of apparent $P_{o/b}$ and EC_{50} for [^3H]TBZOH displacement (Fig. 5). This correlation is described by relation 1 (Eq. 1), which may be formulated in a different way. The compounds following the correlation (TBZ derivatives and substrates) are competitive inhibitors of [^3H]TBZOH binding as previously shown (11, 24) and as indicated by their Hill coefficients (Fig. 2). For these displacers, EC_{50} and K_D values are proportional, according to the Cheng and Prusoff formula (25). Under our experimental conditions, K_D is about 0.6 times the EC_{50} . The correlation between EC_{50} and apparent $P_{o/b}$ can now be formulated as:

$$K_D \times \text{apparent } P_{o/b} = 2.8 \mu\text{M} \quad (2)$$

If we assume, as discussed before, that the apparent $P_{o/b}$ reflects the liposolubility of the compounds, then relation 2 (Eq. 2) suggests that site T equilibrates with the ligand present in the membrane phase, and that this site has the same "intrinsic" affinity for all compounds located on the correlation line

of Fig. 5. The corresponding equilibrium dissociation constant, $K_{D,\text{intrinsic}}$, is related to the apparent K_D by the relation:

$$K_{D,\text{intrinsic}} = K_D \times R_{m/b}$$

where $R_{m/b}$ is the concentration ratio between the buffer and the hydrophobic phospholipid bilayer phase. This ratio can be tentatively derived from $P_{m/b}$ values (see Materials and Methods), and we find for TBZ derivatives and for the natural substrates of uptake an approximate value of 1 μM for $K_{D,\text{intrinsic}}$.

Relation 2 (Eq. 2) thus suggests that all compounds located on the correlation line have the same "intrinsic" affinity for site T, about 1 μM , although their apparent affinities are very different. For these compounds (natural substrates and TBZ derivatives), true differences in affinity cannot be completely ruled out. However, a fortuitous correlation over 5 orders of magnitude is unlikely. The diversity of the apparent dissociation constants may thus be attributed to differences in the concentration ratio between the aqueous and membrane phases.

Activity-structure relationship. The experimental data suggest that 5-HT, adrenaline, noradrenaline, and TBZ derivatives have the same "intrinsic" affinity for site T. Tyramine, which lacks one phenol group, and MIBG, which is neither a catechol nor an amine, have an intrinsic affinity which is decreased by only a factor of 10. The structural differences between these compounds are thus not critical for their specific interaction with the binding site. Accordingly, site T has a broad specificity and recognizes only a few elements of the ligand molecules. An aromatic ring and a basic function precisely located with respect to the aromatic ring may be the only structural requirements. It is interesting to note that the apparent stereoselectivity observed with α and β isomers of TBZOH (Fig. 3) is accounted for by a difference in the apparent $P_{o/b}$ of these isomers (Fig. 5).

Reserpine, haloperidol, and chlorpromazine have very large values of the product $K_D \times \text{apparent } P_{m/b}$. This difference with the other molecules tested (about 3 orders of magnitude) indicates a low intrinsic affinity for site T. For reserpine, this result confirms the existence of a low affinity binding of this compound to site T (12). It should be pointed out that high affinity reserpine binding requires membrane energization by ATP (12, 13), and that about 80% of that binding is lost with the type of preparation used (26). Thus, competition between TBZ and reserpine for binding on their respective high affinity site cannot be analyzed with the type of preparation used. The low "intrinsic" affinity of chlorpromazine and haloperidol may have a different origin. Inhibition of catecholamine uptake and of [^3H]TBZOH binding by these two drugs occurs in the μM range, where a detergent-like action of these compounds has been reported (10). Moreover, it has been suggested that chlorpromazine inhibits catecholamine uptake by affecting the membrane permeability rather than by competitively inhibiting the monoamine transporter (27). Thus, a 3-order of magnitude lower "intrinsic" affinity of chlorpromazine and of haloperidol may simply reflect their nonspecific action.

The fact that site T is in equilibrium with the ligand in the membrane phase might also explain the pH dependence of TBZ and TBZOH apparent affinity (19, 24). The unprotonated form of these amines has a larger apparent $P_{o/b}$ value than the protonated form, thus resulting in an increase with pH of the ligand concentration in the membrane phase. This increase in

turn might account for the apparent increase of affinity of the binding site with the pH.

In conclusion, the apparent high affinity of site T for TBZ may be explained by the hydrophobic character of this site, rather than by structural differences between this drug and the substrates. Our results suggest a localization of the TBZ-binding site inside of the membrane.

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103: 34404v Photoaffinity labeling of the tetrabenazine binding sites of bovine chromaffin granule membranes. Isambert, Marie Francoise; Henry, Jean Pierre (Inst. Biol. Phys.-Chim., Fond. Edmond de Rothschild, 75005 Paris, Fr.). *Biochemistry* 1985, 24(14), 3660-7 (Eng). An azido deriv. of tetrabenazine, a specific inhibitor of the monoamine carrier of chromaffin granule membranes,

was synthesized. In the dark, this compd., ^3H -labeled *N*-(3-laobug-tyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11b//benzo[a]quinolizn-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide (^3H)TBA, bound reversibly to purified chromaffin granule membranes. Centrifugation through SP-Sephadex columns sep'd. bound and free ^3H TBA. This technique gave low levels of nonspecific binding and allowed recovery of ^3H TBA-membrane complexes. Scatchard anal. of the data indicated 1 class of sites with an equil. dissocn. const. K_D of 50 nM and a d. of sites of 40-50 pmol/mg protein, consistent with reported densities of reserpine and dihydrotetrabenazine binding sites. Competition expts. showed that TBA and tetrabenazine bound to the same site. Irradn. at 435 nm of ^3H TBA-membrane mixts. induced some irreversible binding of the probe to membranes. After irreversible binding of TBA, the no. of dihydrotetrabenazine binding sites was decreased, indicating that the probe was covalently bound to the monoamine carrier. ^3H TBA-membrane complexes isolated by centrifugation through SP-Sephadex columns were irradiated, and their radioactivity was analyzed by electrophoresis on SDS-polyacrylamide gels. A polypeptide with a mol. wt. of 70,000 was labeled. This polypeptide was different from dopamine β -hydroxylase, and it was not adsorbed on concanavalin A-Sepharose. It is proposed that the monoamine carrier of chromaffin granule membrane has an oligomeric structure, involving a 45,000-mol.-wt. subunit (Gabizon, R., et al., 1982) and a 70,000-mol.-wt. subunit.

Photoaffinity Labeling of the Tetrabenazine Binding Sites of Bovine Chromaffin Granule Membranes†

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ABSTRACT: An azido derivative of tetrabenazine, a specific inhibitor of the monoamine carrier of chromaffin granule membranes, has been synthesized. In the dark, this compound, ³H-labeled *N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11*bH*-benzo[*a*]quinolizin-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide ([³H]TBA), bound reversibly to purified chromaffin granule membranes. Centrifugation through SP-Sephadex columns was used to separate bound and free [³H]TBA. This technique gave low levels of nonspecific binding and allowed recovery of [³H]TBA-membrane complexes. Scatchard analysis of the data indicated one class of sites with an equilibrium dissociation constant *K*_D of 50 nM and a density of sites of 40–50 pmol/mg of protein, consistent with reported densities of reserpine and dihydrotetrabenazine binding sites. Competition experiments showed that TBA and tetrabenazine bound to the same site. Irradiation at 435 nm of [³H]TBA-membrane mixtures induced some irreversible binding of the probe to membranes. After irreversible binding of TBA, the number of dihydrotetrabenazine binding sites was decreased, indicating that the probe was covalently bound to the monoamine carrier. [³H]TBA-membrane complexes isolated by centrifugation through SP-Sephadex columns were irradiated, and their radioactivity was analyzed by electrophoresis on sodium dodecyl sulfate/polyacrylamide gels. A polypeptide with a molecular weight of 70 000 was labeled. This polypeptide was different from dopamine β-hydroxylase, and it was not adsorbed on concanavalin A-Sepharose. It is proposed that the monoamine carrier of chromaffin granule membrane has an oligomeric structure, involving a 45K subunit [Gabizon, R., Yetinson, T., & Schuldiner, S. (1982) *J. Biol. Chem.* 257, 15145] and a 70K subunit.

Adrenaline and noradrenaline, the catecholamines of adrenal medulla, are stored in specialized organelles, the chromaffin granules, from which they are released by exocytosis. The concentration of catecholamines in chromaffin granules has been estimated to be 0.7 M (Nordmann, 1984), and this high concentration is the result of an active ATP-dependent transport (Kirschner, 1962; Carlsson et al., 1963). The membranes of chromaffin granules contain a carrier that catalyzes an electrodisruptive H⁺/neutral amine antiport (Johnson & Scarpa, 1979; Apps et al., 1980; Scherman & Henry, 1980a; Kanner et al., 1980; Knoch et al., 1980). This carrier accumulates catecholamines in response to the H⁺ electrochemical gradient (inside positive and acidic) generated by an ATP-dependent electrogenic H⁺ pump (Casey et al., 1977; Phillips & Allison, 1978; Johnson & Scarpa, 1979; Scherman & Henry, 1980b). Little is known about the structure of the monoamine carrier. Tetrabenazine (TBZ)¹ and reserpine are specific inhibitors of the carrier (Pletscher, 1976; Scherman & Henry, 1980c). Tritiated derivatives of these drugs have been used in binding studies (Scherman et al., 1983; Scherman & Henry, 1984). [³H]TBZOH binds to chromaffin granule membranes with an equilibrium dissociation constant of 3 nM, and the density of binding sites is 60 pmol/mg of protein, thus indicating that the carrier is not a major component of the membrane (Scherman et al., 1983). [³H]Reserpine binding studies have indicated the presence of a second class of binding sites on the carrier, with a high affinity for reserpine and noradrenaline and no affinity for TBZ (Scherman & Henry, 1984). A purification of the monoamine carrier in a functional state is possible since the

conditions of its solubilization have been defined (Maron et al., 1979; Isambert & Henry, 1981; Scherman & Henry, 1983a). Nevertheless, because of the difficulty of the purification, we have attempted the identification of TBZ binding sites by the technique of photoaffinity. Our probe is *N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11*bH*-benzo[*a*]quinolizin-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide (TBA), a derivative of TBZ coupled to an arylazido moiety. In this paper, we present the results of these photolabeling experiments as well as some methodological improvements to the technique. A communication with a similar aim has appeared in the literature (Gabizon et al., 1982). In this work, the probe was a derivative of 5-HT, a substrate of the carrier.

EXPERIMENTAL PROCEDURES

Chemicals. TBZ and reserpine were obtained from Fluka (Buchs, Switzerland). Sodium cyano[³H]borohydride (98.8 mCi/mg), 1-[7,8-³H]noradrenaline (8 Ci/mmol), and ¹⁴C-methylated protein mixture were obtained from Amersham International (U.K.). [2,3-³H]-γ-Aminobutyric acid was from

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¹ Abbreviations: TBA, *N*-(3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11*bH*-benzo[*a*]quinolizin-2-yl)-4-[(4-azido-2-nitrophenyl)amino]butanamide; TBZ, tetrabenazine (2-oxo-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11*bH*-benzo[*a*]quinolizine); TBZOH, dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11*bH*-benzo[*a*]quinolizine); [³H]TBZOH, [³H]dihydrotetrabenazine; TBZNH₂, 2-amino-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11*bH*-benzo[*a*]quinolizine; NAP-GABA, *N*-(4-azido-2-nitrophenyl)-4-aminobutyric acid; TLC, thin-layer chromatography; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; DBH, dopamine β-hydroxylase.

LA (Saclay, France). Sephadex G-50 (medium) and SP-Sephadex (C-50) were purchased from Pharmacia (Uppsala, Sweden).

Synthesis of TBZNH₂. TBZ (0.3 mmol) and ammonium acetate (1.5 mmol) were incubated with sodium borohydride (0.18 mmol) in 10 mL of methanol (dried on 3-Å molecular sieves) for 4 h at room temperature. TBZNH₂ was purified by TLC on preparative silica gel plates using CHCl₃-methanol (8:2) as the developing solvent and methanol to extract the product from the plates. The two isomers migrated distinctly (*R_f* 0.3 and 0.4), but generally they were not extracted separately. Primary amine reactivity was measured with trinitrobenzenesulfonic acid (Inman & Dintzis, 1969) and was found to be equivalent to the product concentration estimated from UV spectra by using a molecular absorption coefficient $\epsilon_{285} = 3600 \text{ M}^{-1} \text{ cm}^{-1}$ in methanol; yield 50%; mass spectroscopy, $M^+ = 318$; base peak m/z 244 ($M^+ - \text{NH}_3 - \text{isobutyl}$).

Synthesis of [2-³H]TBZNH₂. TBZ (6 μmol) and ammonium acetate (60 μmol) were incubated with 10 mCi (1.6 μmol) of cyano[³H]borohydride in 0.18 mL of dry methanol for 30 min at room temperature. The reaction mixture was purified by HPLC on a C₁₈ $\mu\text{Bondapak}$ column (Waters, Milford, MA) with methanol-10 mM (NH₄)CO₃H (70:30) as the solvent. The two isomers were eluted at 16 (isomer I, 1.1 mCi) and 21 mL (isomer II, 1.3 mCi); they were collected and lyophilized separately. Both had similar specific activity (2.3 Ci/mmol).

Synthesis of TBA. 4-Fluoro-3-nitrophenyl azide was prepared from 4-fluoro-3-nitroaniline (Fleet et al., 1972). It was coupled to 4-aminobutyric acid essentially as described by Levy (1973) to give NAP-GABA. This compound was recrystallized from ethanol. NAP-GABA was activated by *N*-hydroxysuccinimide. Briefly, dicyclohexylcarbodiimide (0.83 mmol) was slowly added to a mixture of NAP-GABA (0.75 mmol) and *N*-hydroxysuccinimide (0.83 mmol) in 20 mL of dry tetrahydrofuran. The mixture was stirred overnight at room temperature. Acetic acid (0.08 mmol) was added, and solids were filtered off. The solution was dried in vacuo over P₂O₅. The product, *N*-succinimidyl 4-[(4-azido-2-nitrophenyl)amino]butyrate, was about 90% pure as judged by TLC on silica gel plates with CHCl₃-methanol (10:1) as the developing solvent. This system separated NAP-GABA (*R_f* 0.23) from the succinimidyl derivative (*R_f* 0.68). The succinimidyl derivative was then coupled with TBZNH₂. In a typical experiment, the activated NAP-GABA derivative (2.4 μmol) and TBZNH₂ (2.0 μmol) were incubated in 0.2 mL of dioxane-H₂O (4:1) for 3 h at room temperature. The reaction mixture was dried under a stream of argon, and the residue was solubilized in methanol and purified by HPLC on a C₁₈ $\mu\text{Bondapak}$ column with methanol-10 mM (NH₄)CO₃H (70:30) as the solvent and a UV detector operating at 260 nm. The coupled product was detected by monitoring the absorption of the azidophenyl moiety and by the radioactivity of tracer amounts of [³H]TBZNH₂. Two products were eluted at 7.8 (compound I) and 9.9 mL (compound II), which contained equivalent amounts of TBZNH₂ and arylazido moieties. Experiments with isolated isomers of TBZNH₂ indicated that the two products were isomers. Compound II (yield 35%) has been used in all subsequent experiments. Its concentration was estimated by using a molecular absorption coefficient $\epsilon_{460} = 5900 \text{ M}^{-1} \text{ cm}^{-1}$ in methanol.

Synthesis of [³H]TBA. (1) [³H]TBZNH₂-Labeled TBA. Isomer II of [³H]TBZNH₂ (0.58 μmol , 1.3 mCi) was reacted with the succinimidyl derivative of TBA (0.6 μmol), and the

resulting [³H]TBA was purified as described above. [³H]TBA (1.9 Ci/mmol, yield 29%) was stored in methanol at -80 °C. Its purity was periodically checked by HPLC.

(2) [³H]GABA-Labeled TBA. 4-Amino-[2,3-³H]butyric acid (1 μmol , 5 mCi) was incubated with 4-fluoro-3-nitrophenyl azide (4 μmol) and triethylamine (4 μmol) in 30 μL of dimethyl sulfoxide at 70 °C overnight. The mixture was lyophilized, and [³H]NAP-GABA was purified by HPLC with methanol-acetic acid-H₂O (60:1:40) as the solvent. [³H]-NAP-GABA (0.6 μmol) was eluted at 11 mL and was lyophilized. [³H]NAP-GABA was incubated with *N*-hydroxysuccinimide (1.2 μmol) and dicyclohexylcarbodiimide (1.2 μmol) in 50 μL of dry tetrahydrofuran at room temperature overnight. The vial was then dried under a stream of argon. TBZNH₂ (2.5 μmol) in 100 μL of tetrahydrofuran-H₂O (3:1) was added, and the mixture was incubated at room temperature. After 3 h, it was dried under argon and purified by HPLC with methanol-30 mM (NH₄)CO₃H (80:20) as the solvent. Isomer II of [³H]TBA (0.12 μmol , 0.57 mCi) was eluted at 10 mL.

In photolabeling experiments, [³H]TBZNH₂-labeled and [³H]GABA-labeled TBAs were tried and gave similar results. Most of the experiments described (with the exceptions of those of Figure 5 and Table II) were performed with the latter compound.

Chromaffin Granule Membrane Preparation. Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6 M sucrose layer. Unless indicated, membranes were frozen in liquid nitrogen and were stored at -80 °C (Smith & Winkler, 1967; Giraudat et al., 1980).

[³H]TBA Binding. Membranes (0.01–1.0 mg of protein/mL) were incubated at room temperature with various concentrations of [³H]TBA in 0.3 M sucrose/20 mM Tris-phosphate buffer (pH 7.0). Usually, bound ligand was measured by centrifugation through SP-Sephadex C-50. The gel was equilibrated in the same buffer, poured into 1-mL plastic syringes, and dehydrated by centrifugation at 500 g for 2 min at room temperature (Penefsky, 1977; Pick & Racker, 1979). Samples (0.2 mL) were added to the dehydrated gel and were centrifuged in the same way. Larger samples (1.0 mL) were centrifuged through 3.0 mL of gel with the same results. When nonspecific binding was measured, 7 μM TBZ was added to the incubation mixture, and the gel was equilibrated in the presence of 10 μM TBZ. In some experiments, filtration through C₁₈ Sep-pak cartridges (Waters, Milford, MA) were used to adsorb free [³H]TBA. Before being used, the cartridge was washed with 2 mL of methanol, 20 mL of H₂O, and 20 mL of buffer. The sample (0.5 mL) was filtered smoothly, and the resin was washed by 1.5 mL of buffer. The yield of [³H]TBA-membrane complex obtained by this technique was low (about 25%); it was increased by filtering three samples of membrane proteins before the [³H]TBA-membrane complex.

SDS/Polyacrylamide Gel Electrophoresis. [³H]TBA-membrane complexes (1 mL) were prepared by centrifugation through SP-Sephadex gels. They were collected in plastic cuvettes and immediately placed in front of a monochromator set at 435 nm (Bausch & Lomb, equipped with an SP-200 mercury light source). Samples were illuminated for 2.5 min at room temperature with stirring. They were diluted to 5 mL with 10 mM Hepes buffer (pH 7.0). The pellets were extracted by 5 mL of ethanol-acetone (1:1), centrifuged at 250000g for 20 min, and solubilized in 0.22 mL of a 50 mM phosphate buffer, pH 7.0, containing 2% SDS, 12% sucrose,

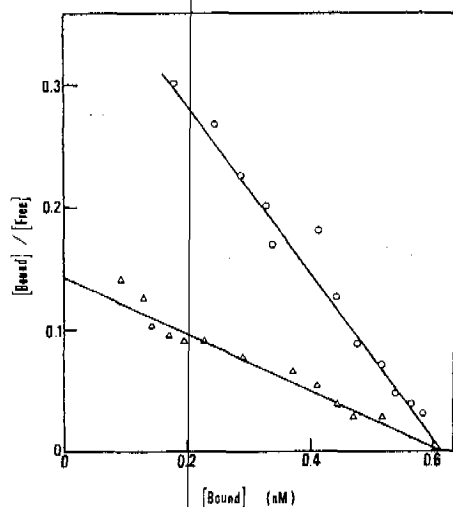


FIGURE 1: Inhibition by TBA of $[^3\text{H}]\text{TBZOH}$ binding. Membranes (0.014 mg of protein/mL) were preincubated in the presence (Δ) or absence (O) of 85 nM TBA for 1 h at room temperature in 1.0 mL of 20 mM Tris-phosphate buffer (pH 7.2) containing 0.3 M sucrose. $[^3\text{H}]\text{TBZOH}$ (0.78–20 nM) was then added, and the mixture was incubated for 90 min at the same temperature. Bound $[^3\text{H}]\text{TBZOH}$ was determined by filtration on Millipore filters. Nonspecific binding was measured by addition of 2 μM TBZ to the incubation mixture and was subtracted. Points are mean of duplicate determinations.

30 mM DTT, and 6 mM EDTA. Aliquots (100–200 μL) were subjected to disc electrophoresis according to Weber & Osborn (1969) on 7.5% polyacrylamide gels for 3 h at 15 mA per gel or to Laemmli (1970) on 8% polyacrylamide gels for 2 h at 4 mA per gel. Ethanol-acetone extraction did not change the electrophoresis pattern (Sutton & Apps, 1981). The gels were then sliced in 2-mm fractions, from which the radioactivity was eluted by 0.8 mL of 6 M urea and 1% SDS (Buisson et al., 1976) and measured by scintillation after addition of 7 mL of Aqualuma (Lumac, Schaesberg, Holland).

Other Techniques. ATP-dependent $[^3\text{H}]\text{noradrenaline}$ uptake was measured essentially as described by Phillips (1974), $[^3\text{H}]\text{TBZOH}$ binding as described by Scherman et al. (1983), and DBH activity as described by Wallace et al. (1973). Proteins were estimated by the Lowry procedure with bovine serum albumin as a standard following precipitation in 5% trichloroacetic acid and redissolution in 2% deoxycholate–3% NaOH. Phospholipid estimation was done by assaying inorganic phosphate liberated by perchloric acid hydrolysis.

RESULTS

TBA and TBZOH Binding Occur on the Same Site. $[^3\text{H}]\text{TBZOH}$ binding has been studied in the presence of a constant concentration of TBA, in the dark (Figure 1). A Scatchard plot of the data indicated that the addition of TBA decreased the binding of $[^3\text{H}]\text{TBZOH}$, without affecting the number of binding sites B_{max} . This result indicates that the two drugs compete for the same site. From these data, the equilibrium dissociation constant for the binding of TBA was also calculated and was found to be 47 nM. The two drugs thus bind to the same site, and the affinity of this site for TBA is an order of magnitude lower than for TBZOH.

Centrifugation through Sephadex Gels as an Assay of $[^3\text{H}]\text{TBA}$ Binding in the Dark. The direct binding of $[^3\text{H}]\text{TBA}$ to purified chromaffin granule membranes was difficult to investigate by the classical filtration technique, because of the high background due to ligand adsorption on cellulose or glass-fiber filters. Therefore, the bound complex was separated

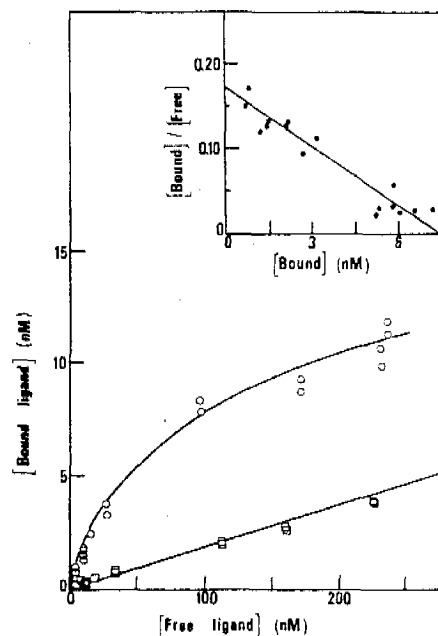


FIGURE 2: Saturation isotherm obtained by filtration of the ligand through SP-Sephadex columns. Membranes (0.15 mg of protein/mL) were incubated with $[^3\text{H}]\text{TBA}$ (5–500 nM) in the absence (O) or presence ($\square$) of 5 μM TBZ for 40 min at room temperature in 0.2 mL of 20 mM Tris-phosphate buffer (pH 7.0) containing 0.3 M sucrose. Inset: Scatchard plot of the data.

from the free ligand by low-speed centrifugation through Sephadex gels (Penefsky, 1977; Pick & Racker, 1979). In this technique, the gel is dehydrated by a previous centrifugation under the same conditions, thus suppressing dilution of the sample. We found that the negatively charged SP-Sephadex gave better retention of free $[^3\text{H}]\text{TBA}$ than did the neutral Sephadex G-50. In the absence of granule membranes, 2% or less of the layered radioactivity was recovered in the void volume, whereas under the same conditions of centrifugation, the recovery of the membrane protein was higher than 90%. To evaluate the validity of the centrifugation technique for the quantitative estimation of bound ligands, a saturation isotherm of the binding of $[^3\text{H}]\text{TBZOH}$ was performed by this technique. A Scatchard plot of the data indicated a dissociation equilibrium constant K_D of 2.0 nM and a density of sites B_{max} of 27 pmol/mg of protein, which should be compared with values of 1.4 nM and 45 pmol/mg of protein for respectively K_D and B_{max} obtained by filtration on Millipore filters (Figure 1). The technique of filtration through SP-Sephadex gels has thus been utilized to study $[^3\text{H}]\text{TBA}$ binding (Figure 2). The binding observed in the presence of 7 μM TBZ has been considered as nonspecific and has been subtracted to estimate specific binding. A saturation isotherm obtained by this technique is given in Figure 2. Analysis of the data (Figure 2, Inset) showed one class of binding sites characterized by a K_D of 43 nM and a B_{max} of 50 pmol/mg of protein.

Characteristics of $[^3\text{H}]\text{TBA}$ Binding in the Dark. At room temperature, the equilibrium was reached rapidly, about 20 min being required at a concentration of 100 nM. The equilibrium was temperature dependent; the level of $[^3\text{H}]\text{TBA}$ binding observed at 22 $^{\circ}\text{C}$ decreased by a factor of 2 after a 10-min incubation at 0 $^{\circ}\text{C}$. The kinetics of dissociation were measured by addition of 8 μM TBZ to membranes preincubated with 80 nM $[^3\text{H}]\text{TBA}$. In this experiment, free and bound ligands were separated by rapid filtration on small

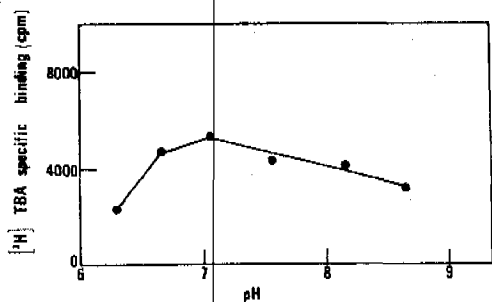


FIGURE 3: pH dependency of $[^3\text{H}]\text{TBA}$ binding. Membranes (0.6 mg of protein/mL) were incubated with $[^3\text{H}]\text{TBA}$ (60 nM) in 0.5 mL of 0.3 M sucrose/20 mM Tris-phosphate buffers at various pH, in the absence or presence of 8 μM TBZ. Duplicate determinations of bound $[^3\text{H}]\text{TBA}$ were performed by centrifugation through SP-Sephadex columns equilibrated at the pH of the incubation mixture. Centrifugation through Sephadex columns and filtration through C_{18} Sep-pak cartridges gave similar pH profiles of the specific radioactivity.

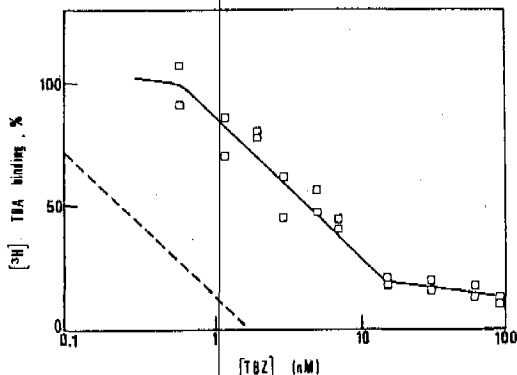


FIGURE 4: Displacement of $[^3\text{H}]\text{TBA}$ by TBZ. Membranes (0.5 mg of protein/mL) were preincubated with TBZ (0–90 nM) for 18 h at room temperature in 0.5 mL of 20 mM Tris-phosphate buffer (pH 7.2) containing 0.3 M sucrose. $[^3\text{H}]\text{TBA}$ (10 nM) was then added, and the mixture was incubated for 2 h at the same temperature. Bound $[^3\text{H}]\text{TBA}$ was determined by centrifugation of 0.2-mL aliquots on SP-Sephadex columns. The dotted line indicates the theoretical slope at midpoint, assuming a Hill number of 1.0.

C_{18} -bonded SiO_2 cartridges, which adsorbed the free ligand. The half-lifetime measured at pH 8.2 and at pH 7.0 is 7 min. $[^3\text{H}]\text{TBZOH}$ which has a pK_A of 7.5 (Scherman & Henry, 1982) has a K_D decreasing with increasing values of the pH, up to pH 8.0 (Scherman & Henry, 1983b). This pH dependency has been attributed to the fact that the monoamine carrier binds only the neutral form of the drug. The effect of the pH on $[^3\text{H}]\text{TBA}$ binding has been studied at a drug concentration of 60 nM (Figure 3). Contrasting with $[^3\text{H}]\text{TBZOH}$, $[^3\text{H}]\text{TBA}$ binding had a broad pH optimum around pH 7.0. Saturation isotherms had similar characteristics at pH 7.0 and 8.0 (K_D = 43 nM and B_max = 50 pmol/mg of protein at pH 7 and K_D = 56 nM and B_max = 60 pmol/mg of protein at pH 8).

The specificity of $[^3\text{H}]\text{TBA}$ binding is supported by displacement experiments. $[^3\text{H}]\text{TBA}$ is displaced from its binding site by TBZ (Figure 4). The slope of the curve indicates a competition between the two drugs, thus confirming the results of the experiment of Figure 1. The EC_{50} derived from this experiment is 4 nM, from which a K_D of 3 nM for TBZ can be derived. This figure is consistent with that previously obtained (Scherman et al., 1983). $[^3\text{H}]\text{TBA}$ is also displaced from its binding site by TBA, reserpine, and 5-HT (Table I). The equilibrium dissociation constants for reserpine and TBZ derived from these experiments are similar to those previously

Table I: Displacement of $[^3\text{H}]\text{TBA}$ in the Dark^a

compounds	EC_{50} (nM)	K_D (nM) ^b
TBZ	3.8	2.9 (1.3)
TBA	40	31
reserpine	55	42 (25)
5-HT	360×10^3	277×10^3 (240×10^3)

^a Experimental conditions were as described in Figure 4. ^b K_D values were calculated from EC_{50} as described by Jacobs et al. (1975). Figures in parentheses are derived from Scherman et al. (1983) and Scherman & Henry (1984).

Table II: Irreversible Binding of $[^3\text{H}]\text{TBA}$ after Irradiation

membrane treatment	incubations ^a		radioactivity specifically bound ^b (pmol/mL)
	membranes + $[^3\text{H}]\text{TBA}$ (pmol/mL)	membranes + TBZ + $[^3\text{H}]\text{TBA}$ (pmol/mL)	
incubation in the dark ^c	260	110	150
incubation and extraction in the dark ^d	3	3	0
incubation, irradiation, and extraction ^e	34	14	20

^a Membranes (0.96 mg of protein/mL) were incubated with $[^3\text{H}]\text{TBA}$ (1 μM) in the absence or presence of 8 μM TBZ for 45 min. ^b Calculated as the difference between columns 2 and 3. ^c Bound $[^3\text{H}]\text{TBA}$ was determined by centrifugation of 0.2-mL aliquots through SP-Sephadex gels. ^d Samples centrifuged through SP-Sephadex columns were extracted by 1.6 mL of ethanol-acetone (1:1) and centrifuged at 10000g for 50 min; the pellets were solubilized in 0.25 mL of 2% deoxycholate-3% NaOH, and their radioactivity was measured by liquid scintillation in Aqualuma. ^e Samples centrifuged through SP-Sephadex columns were rapidly transferred in front of the monochromator set at 435 nm and irradiated for 2.5 min. The membranes were then extracted by ethanol-acetone as in footnote d.

reported (Scherman & al., 1983; Scherman & Henry, 1984).

Irreversible Binding of $[^3\text{H}]\text{TBA}$ in the Light. When $[^3\text{H}]\text{TBA}$ -membrane complexes isolated in the dark by filtration on SP-Sephadex gels were extracted by an ethanol-acetone mixture (1:1), the specifically bound radioactivity was completely removed (Table II, line 2). This extraction removed 75% of the membrane phospholipid but did not extract any protein (Sutton & Apps, 1981). If the $[^3\text{H}]\text{TBA}$ -membrane complex was rapidly irradiated at 435 nm after its isolation, then some specific radioactivity was not extractable by acetone-ethanol (Table II, line 3). This formation of an irreversible derivative was very reproducible, with a yield of $23 \pm 6\%$ (mean $\pm$ SD, $n = 16$). The same result was observed with $[^3\text{H}]\text{TBZNH}_2$ labeled as well as with $[^3\text{H}]\text{GABA}$ -labeled TBA. The kinetics of this reaction were rapid and under the conditions used, it was terminated in less than 4 min (Figure 5).

It was important to verify that the irreversible binding occurred on the same site as the reversible one. Advantage was taken from the fact that TBA and $[^3\text{H}]\text{TBZOH}$ compete for the same sites (Table III, lines 1 and 2). TBA can be washed out by dilution and centrifugation, thus restoring $[^3\text{H}]\text{TBZOH}$ binding to its initial value (Table III, lines 3 and 4). If the mixture of TBA and membranes was irradiated before the washing step, a 20–30% inhibition of $[^3\text{H}]\text{TBZOH}$ binding was observed (Table III, line 5).

Analysis of the $[^3\text{H}]\text{TBA}$ -Labeled Membranes by Electrophoresis on SDS/Polyacrylamide Gels. As a first attempt to identify $[^3\text{H}]\text{TBA}$ binding sites, mixtures of $[^3\text{H}]\text{TBA}$ and membranes were irradiated at 435 nm, and the proteins were analyzed by gel electrophoresis. By this technique no polypeptide chain was selectively labeled. This negative result was

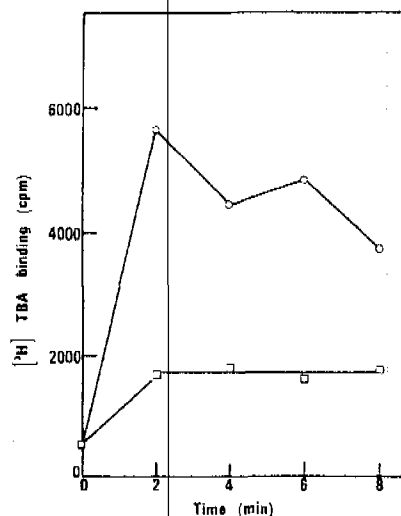


FIGURE 5: Kinetics of the effect of illumination of $[^3\text{H}]\text{TBA}$ -membrane complexes. Membranes (0.76 mg of protein/mL) were incubated with $[^3\text{H}]\text{TBA}$ (340 nM) in the absence (O) or in the presence (□) of 15 μM TBZ in 0.3 M sucrose/20 mM Tris-phosphate buffer (pH 8.15). Aliquots (0.225 mL) were centrifuged through SP-Sephadex columns, rapidly transferred in front of the monochromator set at 435 nm, and irradiated for the indicated period of time. The membranes were then extracted by ethanol-acetone and treated as described in the footnotes to Table II.

Table III: TBA Binds Covalently to $[^3\text{H}]\text{TBZOH}$ Binding Sites

	specific $[^3\text{H}]\text{TBZOH}$ binding ^a (cpm)	inhibition ^b (%)
(1) membranes ^c	3570 $\pm$ 260 ($n = 3$)	control
(2) membranes + TBA ^c	1210 $\pm$ 90 ($n = 2$)	66
(3) washed membranes ^d	2180 $\pm$ 100 ($n = 4$)	control
(4) membranes + TBA, washed ^d	2130 $\pm$ 250 ($n = 4$)	2
(5) membranes + TBA, irradiated, washed ^d	1620 $\pm$ 90 ($n = 4$)	26

^a $[^3\text{H}]\text{TBZOH}$ (2 nM) binding was measured on membrane samples diluted 10 times in 0.3 M sucrose/Tris-phosphate buffer (pH 7.0) at about 0.01 mg of protein/mL. Nonspecific binding measured in the presence of 2 μM TBZ was subtracted. Measurements are mean $\pm$ SD. Similar results have been obtained in two independent experiments. ^b It has been verified that irradiation of the membranes alone did not change $[^3\text{H}]\text{TBZOH}$ binding. ^c Membranes (0.09 mg protein/mL, 1 mL final volume) were incubated without (line 1) or with (line 2) 1.8 μM TBA. ^d Membranes were incubated without (line 3) or with (lines 4 and 5) TBA (1.8 μM) and in line 5, were illuminated at 435 nm for 2.5 min (1 mL final volume). They were then diluted 10 times in sucrose/Tris-phosphate buffer, and after incubation for 10 min at room temperature and 30 min at 0 $^{\circ}\text{C}$, they were centrifuged for 30 min at 250000g. Pellets were resuspended in 1 mL of the same buffer, and after a 10-fold dilution they were assayed for $[^3\text{H}]\text{TBZOH}$ binding. The protein concentrations of the resuspended pellets were equivalent and were 0.05 mg of protein/mL.

explained by the intensity of $[^3\text{H}]\text{TBA}$ nonspecific binding (Table IV). The level of nonspecific binding was reduced when $[^3\text{H}]\text{TBA}$ -membrane complexes isolated by centrifugation on SP-Sephadex were immediately irradiated (Table IV). Electrophoresis of such complexes revealed a clear peak of radioactivity (Figure 6). No radioactivity peak was observed when 7 μM TBZ or 1 mM 5-HT was added to the incubation mixture before irradiation. Calibration of the gel with ^{14}C -methylated protein markers indicated a molecular weight of 70000 for the labeled polypeptide. In the experiment of Figure 6, the irradiated complex had been extracted with acetone-ethanol (1:1) prior to being electrophoresed. When this step was omitted, a large amount of radioactivity was found in the front of the gel (Figure 7). This radioactivity

Table IV: Decrease of $[^3\text{H}]\text{TBA}$ Nonspecific Binding by Centrifugation on SP-Sephadex Gels

	bound radioactivity ^a (pmol/mL)		specific binding
	-TBZ	+TBZ	
reversible binding, ^b membranes + $[^3\text{H}]\text{TBA}$	21.2	4.5	16.7
irreversible binding, ^c irradiation of membranes + $[^3\text{H}]\text{TBA}$ ^d	10.2	8.2	2.0
irradiation of the isolated $[^3\text{H}]\text{TBA}$ -membrane complex ^e	3.1	0.6	2.5

^a The columns give total binding (column 1), nonspecific binding determined in the presence of 8 μM TBZ (column 2) and specific binding obtained as the difference between columns 1 and 2. ^b Membranes (1.0 mg of protein/mL) were incubated with $[^3\text{H}]\text{TBA}$ (65 nM) in 0.3 M sucrose/20 mM Tris-phosphate buffer (pH 7.0). Reversible binding was measured by centrifugation through SP-Sephadex columns. ^c The irradiated samples were washed and extracted by ethanol-acetone, as described for gel electrophoresis. ^d Irradiation of $[^3\text{H}]\text{TBA}$ membrane mixtures for 2.5 min at 435 nm. Gel electrophoresis of these samples indicated multiple radioactivity peaks with no clear displacement by TBZ. ^e Irradiation of $[^3\text{H}]\text{TBA}$ membrane complexes isolated by centrifugation on SP-Sephadex gels as described under Experimental Procedures. Gel electrophoresis of these samples indicated a major peak of radioactivity with a molecular weight of about 65000.

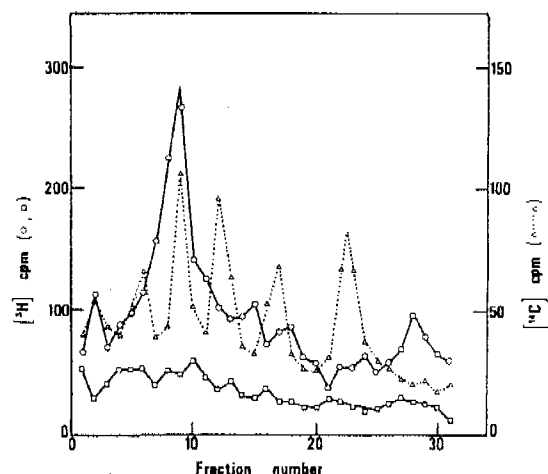


FIGURE 6: SDS/polyacrylamide gel electrophoresis of irradiated $[^3\text{H}]\text{TBA}$ -membrane complexes. Membranes (0.8 mg of protein/mL) were incubated with $[^3\text{H}]\text{TBA}$ (100 nM) in the absence (O) or presence (□) of 7 μM TBZ in 0.3 M sucrose/20 mM Tris-phosphate buffer (pH 7.0). Samples (1.3 mL) were processed as described under Experimental Procedures. ^{14}C -Labeled protein markers (Δ) were mixed with the $[^3\text{H}]\text{TBA}$ sample before electrophoresis. Electrophoresis was performed according to Weber & Osborn (1969). Marker molecular weights were the following: myosin, 200K; phosphorylase b, 92.5K; bovine serum albumin, 69K; ovalbumin, 46K; carbonic anhydrase, 30K; lysozyme, 14.3K.

is likely to be due to free or lipid-bound $[^3\text{H}]\text{TBA}$ derivatives. However, the possibility of the labeling of a minor peptide of low molecular weight has not been investigated. Use of chromaffin granule membranes prepared in the presence of protease inhibitors (5 $\mu\text{g}/\text{mL}$ soybean trypsin inhibitor, 100 $\mu\text{g}/\text{mL}$ bacitracin, 1 mM benzamide, 0.1 mM EDTA, 0.1 mM phenylmethanesulfonyl fluoride) did not change the electrophoretic pattern. In addition, the same 70K polypeptide was labeled when the experiment was performed immediately after preparation of the membranes, on nonfrozen material.

We have attempted to decrease the duration of the time required for the isolation of $[^3\text{H}]\text{TBA}$ -membrane complex.

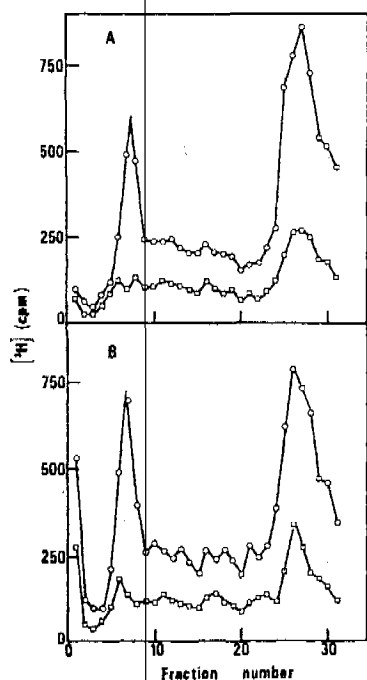


FIGURE 7: Gel electrophoresis of reduced (A) or nonreduced (B) $[^3\text{H}]$ TBA-membrane complexes. Experimental conditions were similar to those of figure 6, but irradiated membranes were not extracted by acetone-ethanol. The washed membranes were solubilized by the described SDS buffer with (A) or without (B) 30 mM DTT. The molecular weight of the $[^3\text{H}]$ TBA-labeled peaks was about 70 000.

The best results have been obtained by rapid filtration of $[^3\text{H}]$ TBA-membrane mixtures through small SiO_2 cartridges, directly in a cuvette in front of the monochromator. The electrophoretic patterns of samples treated in this way indicated a radioactivity peak with a molecular weight of 70 000 and a low background. We have also noted that $[^3\text{H}]$ TBA probes tritiated on the TBZ or the GABA moiety labeled the same 70K polypeptide.

$[^3\text{H}]$ TBA-Labeled Polypeptide Is Not Dopamine β -Hydroxylase (DBH). DBH is the major component of chromaffin granule membrane (Hornagel et al., 1972). In the presence of reducing agents, it migrates on SDS/polyacrylamide gels as a monomer with a molecular weight of 75 000 (Wallace et al., 1973). It was thus of importance to verify that our results did not originate in some low-affinity interaction between $[^3\text{H}]$ TBA and this protein. TBA (up to 0.7 μM) and TBZ (2.4 μM) were not inhibitors of DBH activity (data not shown). Moreover, in the absence of reducing agents, DBH migrates as a dimer with a molecular weight of 150 000. This shift from M_r 75 000 to 150 000 is considered to be characteristic of DBH (Cahill & Morris, 1979). Omission of the reducing agent did not change the mobility of the $[^3\text{H}]$ TBA-labeled component (Figure 7).

Finally, in another set of experiments, advantage was taken from the fact that DBH is a glycoprotein with a high affinity for concanavalin A. $[^3\text{H}]$ TBA-labeled membranes were solubilized by low SDS concentrations, and the soluble components were filtered through a concanavalin A-Sepharose column, according to Fischer-Colbrie et al. (1982). A large fraction (75%) of the radioactivity was not retained on the column, and analysis by electrophoresis on polyacrylamide gels indicated that this fraction was associated with the 70K polypeptide (Figure 8). In the same experiment, the densitometric scanning of the protein stained with Coomassie blue is shown. From the comparison of the two profiles, it is obvious

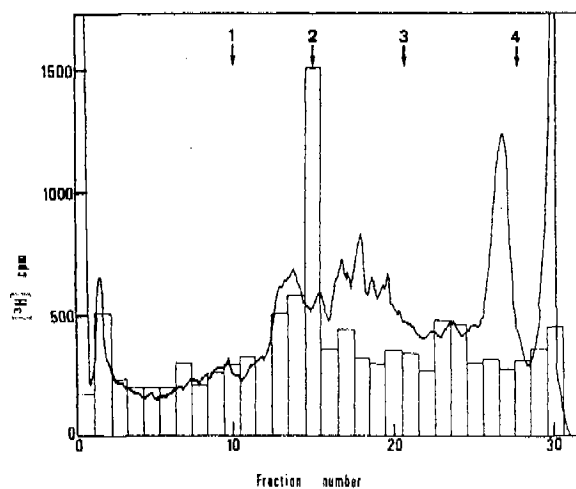


FIGURE 8: Gel electrophoresis of membranes labeled with $[^3\text{H}]$ TBA and filtered through concanavalin A-Sepharose. Membranes (0.5 mg of protein/mL) were incubated with 70 nM $[^3\text{H}]$ TBA in 6.0 mL of 0.3 M sucrose/20 mM Tris-phosphate buffer (pH 7.2). The sample was centrifuged through SP-Sephadex gels, irradiated, and washed as described under Experimental Procedures. The pellet was solubilized by 2.0 mL of 0.07% SDS in 100 mM NaCl/100 mM phosphate buffer (pH 7.4). The mixture was centrifuged at 160 000g for 45 min. The supernatant was filtered through concanavalin A-Sepharose gel as described by Fischer-Colbrie et al. (1982). The filtrate was delipidated and concentrated according to Wessel et al. (1984). The samples were then analyzed by electrophoresis according to Laemmli (1970). The electrophoretogram shows the radioactivity of gel slices and a densitometric scan of Coomassie stained protein. Arrows indicate molecular weight markers: 1, phosphorylase b; 2, bovine serum albumin; 3, ovalbumin; 4, carbonic anhydrase.

that $[^3\text{H}]$ TBA specifically labels a minor component of the membrane.

DISCUSSION

We describe the synthesis of a probe, TBA, intended to label the TBZ binding site. The binding in the dark of $[^3\text{H}]$ TBA to chromaffin granule membranes has been followed by the technique of centrifugation on Sephadex gels, originally proposed by Pick (Penefsky, 1977). This technique has several advantages over the classical filtration assay. The membrane-ligand complex is obtained in solution and can thus be easily manipulated. Moreover, the level of nonspecific binding is low. In several experiments, we have verified that it was not very different from the radioactivity recovered in the absence of granule membranes, thus suggesting that nonspecific binding controls contained principally free ligand. Nevertheless, the reliability of the technique from the quantitative point of view has to be assessed. From binding data obtained at different $[^3\text{H}]$ TBA concentrations and assuming a K_D of 50 nM, we can calculate a density of sites B_{max} of 38 ± 19 pmol/mg of protein (SD, $n = 26$). This figure can be considered as being in good agreement with that obtained by filtration for $[^3\text{H}]$ TBZOH binding sites (Scherman et al., 1983).

$[^3\text{H}]$ TBA binds in the dark to the same site than TBZ since (i) both drugs have similar densities of sites (Figures 1 and 2), (ii) TBA inhibits competitively the binding of $[^3\text{H}]$ TBZOH (Figure 1), (iii) TBZ displaces $[^3\text{H}]$ TBA from its sites (Figure 4), and (iiii) the dissociation constants of TBZ, reserpine, and 5-HT derived from $[^3\text{H}]$ TBA displacement experiments are similar to those derived from $[^3\text{H}]$ TBZOH displacement ones (Table I). The derivatization of TBZ to TBA has thus only decreased the affinity of this new compound for chromaffin granule membrane. This change of affinity may be the result

of the introduction of an extra amino group in the TBZ moiety since TBZNH₂ has an affinity of the same order as TBA, as judged from [³H]TBZOH displacement and noradrenaline uptake inhibition experiments (data not shown). This amino group is likely to have a pK_a higher than 7.5, the pK_a of the ring tertiary amine of TBZOH (Scherman & Henry, 1982). The observed affinity decrease might thus result from the presence of a positive charge in the TBZ moiety, a hypothesis consistent with the fact that the carrier binds only the neutral form of substrate and inhibitor monoamines (Scherman & Henry, 1983b). Though we have not systematically investigated the acid–basic properties of TBA, we have noted a difference in the pH dependency of [³H]TBZOH and [³H]-TBA binding. Increasing the pH from 7.0 to 8.0 decreases the K_D of the former drug from 7.0 to 4.7 nM (Scherman & Henry, 1983b) and did not change that of the latter one.

[³H]TBA binding in the dark is reversible. This is shown by (i) the displacement of [³H]TBA by TBZ (Figure 4), (ii) direct measurement of the rate of dissociation, and (iii) the fact that the ligand can be extracted by organic solvents (Table II) or washed away (Table III). Nevertheless, bound [³H]-TBA can be separated from free ligand by rapid centrifugation through SP-Sephadex columns since the half-lifetime of the complex (7 min) is larger than the time required to isolate it (2 min). Under the conditions used (50–100 nM [³H]TBA), it can be calculated that 80% of the particulate ligand is specifically bound and that 80% of that fraction is present as a membrane–ligand complex after centrifugation through SP-Sephadex. These conditions are thus suitable to get high levels of specific labeling. Irradiation of isolated [³H]TBA complexes gives rise to some irreversible binding of the probe. Irreversibly bound [³H]TBA is washed neither by dilution and centrifugation (Table III) nor by organic solvents (Table II). Irreversible binding labels a polypeptide with a molecular weight of 70K (Figure 6). The labeling is considered to be specific for the following reasons: (i) Irradiation of TBA–membrane complexes decreases the number of [³H]TBZOH binding sites and the number of [³H]TBZOH binding sites photoinactivated is similar to the yield of [³H]TBA irreversible binding (Table III). This result argues against a transfer of the probe to a protein of the vicinity during isolation of [³H]TBA–membrane complexes. Such an artifact is also ruled out by the fact that the isolation time can be decreased to less than 15 s by filtration through SiO₂ cartridges, without affecting the labeling of the 70 k polypeptide. (ii) The peak of radioactivity at 70K is not observed when TBZ of 5-HT was added to the incubation mixture before irradiation. In the experiment of Figure 7, the concentration of TBZ is 7 μM, but similar results were obtained with a 500 nM drug concentration. (iii) A comparison of the densitometric scanning of the protein stained with Coomassie blue and of the [³H]-TBA radioactivity profile (Figure 8) indicates that the probe labels a minor component of the membrane and is not associated in a nonspecific manner with the peptidic material. Since the major protein of chromaffin granule membrane, DBII, has a molecular weight of 75K, care has been taken to demonstrate that [³H]TBA does not label DBII as a result of nonspecific or low-affinity binding. Therefore, we propose that the TBZ binding site is borne by a 70K polypeptide.

Previous work from this laboratory (Scherman et al., 1983) has indicated that the TBZ binding site is associated with the monoamine transporter, and it can thus be proposed that the TBA-labeled 70K polypeptide is the transporter or one of its subunits. Nevertheless, Gabizon et al. (1982), using a different probe, ANPA-5-HT, derived from 5-HT, have labeled a 45K

polypeptide by the same technique. Experiments are now in progress to demonstrate that the TBA-labeled polypeptide is directly associated with amine transport. This hypothesis is interesting since it suggests an oligomeric structure for the monoamine carrier of chromaffin granules. Since the two probes are different, it can be proposed that the 70K subunit carries TBZ binding sites, whereas the 45K one has a site of high affinity for the substrate. In a previous communication (Scherman & Henry, 1984), it has been proposed that the carrier has two monoamine binding sites: sites R₁ of high affinity for substrates and reserpine and with no affinity for TBZ and sites R₂ with a high affinity for TBZ and low affinities for substrates and reserpine. We can now speculate that R₁ sites are borne by the 45K subunit whereas R₂ sites belong to that of 70K.

ACKNOWLEDGMENTS

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Registry No. TBA, 96557-41-4; [2-³H]TBZNH₂-labeled TBA, 96557-37-8; [2,3-³H]GABA-labeled TBA, 96557-38-9; TBZNH₂, 96614-27-6; NAP-GABA, 58775-36-3; [2-³H]TBZNH₂, 96557-39-0; [2,3-³H]NAP-GABA, 96557-40-3; tetrabenazine, 58-46-8; N-succinimidyl 4-[(4-azido-2-nitrophenyl)amino]butyrate, 62618-07-9; 4-amino[2,3-³H]butyric acid, 13048-68-5; 4-fluoro-3-nitrophenyl azide, 28166-06-5.

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Photoaffinity Labeling of the Pactamycin Binding Site on Eubacterial Ribosomes[†]

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ABSTRACT: Pactamycin, an inhibitor of the initial steps of protein synthesis, has an acetophenone group in its chemical structure that makes the drug a potentially photoreactive molecule. In addition, the presence of a phenolic residue makes it easily susceptible to radioactive labeling. Through iodination, one radioactive derivative of pactamycin has been obtained with biological activities similar to the unmodified drug when tested on *in vivo* and cell-free systems. With the use of [¹²⁵I]iodopactamycin, ribosomes of *Escherichia coli* have been photolabeled under conditions that preserve the activity of the particles and guarantee the specificity of the binding sites. Under these conditions, RNA is preferentially labeled when free, small ribosomal subunits are photolabeled, but proteins are the main target in the whole ribosome. This indicates that an important conformational change takes place in the binding site on association of the two subunits. The major labeled proteins are S2, S4, S18, S21, and L13. These proteins in the pactamycin binding site are probably related to the initiation step of protein synthesis.

The study of the mode of action of antibiotics has the 2-fold interest of opening up possibilities to potentiate their therapeutic activity and of providing important data that can help us to understand the structure and activity of their biological targets.

Pactamycin, a drug initially developed as a potential anti-tumor agent, is especially interesting as an inhibitor of protein synthesis in eukaryotic and prokaryotic systems (Goldberg, 1974). It blocks the initial steps of the protein synthesis process [for a review, see Vázquez (1979)] either by interfering with the association of the large ribosomal subunit with the 40S initiation complex or by blocking the ribosome after the formation of the first peptide bond (Kappen & Goldberg, 1976). In any case, the drug binds preferentially to the small subunit and has lower affinity for the whole ribosome and practically none for the large subunit when tested at low inhibitory concentrations (McDonald & Goldberg, 1970).

The identification of the ribosomal components involved in the antibiotic binding site is an important step in understanding its mode of action. Affinity labeling techniques are probably the most direct method to approach this problem and, in fact, have been used fruitfully in the case of many ribosomal ef-

factors (Cooperman, 1980). The applicability of this technique is usually restricted to cases where radioactively labeled and chemically reactive derivatives are available. Pactamycin, having in its molecule a potentially photoactive (Cowan & Drisko, 1978) acetophenone group, as well as a phenolic group susceptible to radioactive iodination, has the appropriate conditions for the identification of the components of its ribosomal binding site using photoaffinity procedures.

The identification of these components in *Escherichia coli* ribosomes would allow us, considering the activity of the drug, to establish correlations between ribosomal components involved, directly or indirectly, in the initiation step of protein synthesis.

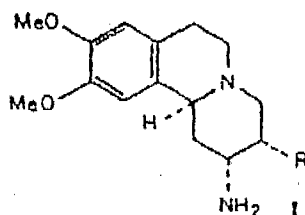
MATERIALS AND METHODS

Iodination of Pactamycin. Pactamycin (25–50 µg) in 50 mM phosphate, pH 7.5, and 50% ethanol was treated with 0.5–0.6 mCi of Na¹²⁵I by a modification of the chloramine T method that avoids direct contact between the oxidant compound and the drug, as reported previously (Tejedor & Ballesta, 1982).

After treatment, the reaction mixture was separated by an LH20 Sephadex column (0.9 × 20 cm), equilibrated, and eluted with 96% ethanol. Fractions (0.5 mL) were collected and checked in a γ counter. The fractions containing the drug were pooled, evaporated, dissolved in 200 µL of Cl₂CH₂, and

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43232e Synthesis of 2-amino-9,10-dimethoxyhexahydrobenzo[a]quinolizines.
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Merour, Anne; Melon, Jean Marie; Champagnac, Andre;
Pommier, Michele; Seine, Christiane (Lab. Chim. Org. V,

Fac. Sci., Orleans, Fr.). *Chim. Ther.* 1972, 7(5), 404-7 (Fr).
The 2-amino-9,10-dimethoxyhexahydrobenzo[a]quinolizines I
(R = H, Me, CH₂CHMe₂) were prepd. from the corresponding
benzo[a]quinolizin-2-ones via the alics., their tosylates, and
azides. The β -amino epimers of I were obtained by reducing the
corresponding oximes. I had slight sedative and antispasmodic
activity. Increase in the size of R increased both the toxicity and
activity of I. The β -epimers were as toxic as I but practically
devoid of activity.

Synthèse d' amino-2 hexahydro benzo-[a]quinolizines

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(Manuscrit reçu le 11 octobre 1971.)

Les deux épimères des amino-2 diméthoxy-9,10 hexahydro-1,2,3,4,6,7 11b H benzo[a]quinolizines substitués ou non en 3 ont été préparés en vue d'une étude pharmacologique comparative. Les épimères α ont été obtenus par une méthode stéréospécifique, les épimères β par réduction des oximes correspondantes. Ces molécules possèdent une faible activité sédative, antispasmodique et vasodilatatrice.

Introduction.

L'intérêt biologique du squelette benzo[a]quinolizine a suscité de nombreux travaux. P. BITE et collaborateurs (1), en particulier, dans le but de synthétiser quelques dérivés N-acylés, ont préparé différentes amino-2 benzo[a]quinolizines par réduction des composés hydroxyimino-2 correspondants.

Nous nous sommes proposés de préparer les deux stéréoisomères α et β des amino-2 diméthoxy-9,10 hexahydro-1,2,3,4,6,7 11b H benzo[a]quinolizines substitués ou non en 3 afin d'étudier du point de vue pharmacologique :

- la relation existant entre la configuration et l'activité,
- l'influence du substituant en 3.

Partie chimique.

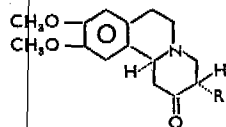
La réduction des oximes 16, 17 et 18 par l'hydrure de lithium et d'aluminium (1) conduit à un mélange des deux épimères. Il était donc nécessaire de mettre au point une préparation stéréospécifique de chacune des amines.

Une méthode réputée telle d'introduction du groupe amino consiste en l'attaque du tosylate de l'alcool correspondant par l'azoture de sodium dans un solvant aprotique selon une réaction à caractère SN_2 prédominant, suivie de la réduction du dérivé azido (2, 3).

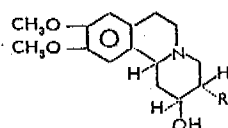
L'amine primaire ainsi obtenue possède la configuration inverse de l'alcool de départ.

Préparation des dérivés amino-2 (a) α .

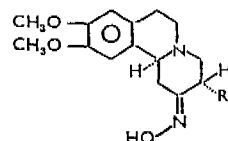
La réduction des cétones 1, 2 et 3 par différentes méthodes (4), nous a conduits exclusivement aux alcools (ϵ) β 4, 5 et 6, dont les tosylates 7, 8 et 9, obtenus par action du chlorure de tosylo dans la pyridine à basse température, traités par l'azoture de sodium dans l'hexaméthylphosphotriamide (HMPT) nous ont donné les azides (α) 10, 11 et 12.



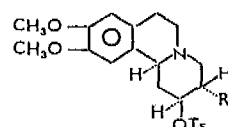
- 1 R = H
2 R = CH₃
3 R = CH₂ - CH(CH₃)₂



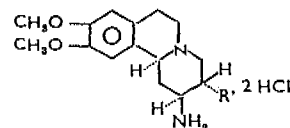
- 4 R = H
5 R = CH₃
6 R = CH₂ - CH(CH₃)₂



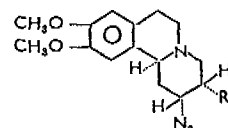
- 16 R = H
17 R = CH₃
18 R = CH₂ - CH(CH₃)₂



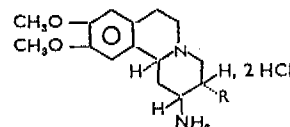
- 7 R = H
8 R = CH₃
9 R = CH₂ - CH(CH₃)₂



- 19 R = H
20 R = CH₃
21 R = CH₂ - CH(CH₃)₂



- 10 R = H
11 R = CH₃
12 R = CH₂ - CH(CH₃)₂



- 13 R = H
14 R = CH₃
15 R = CH₂ - CH(CH₃)₂

La réduction de 10, 11 et 12, soit par voie catalytique, soit par LiAlH_4 , conduit aux dérivés amino-2 α cherchés 13, 14 et 15.

Préparation des dérivés amino-2 (e) β .

La réduction des cétones ne nous permettant pas d'obtenir les alcools 2 α , nous avons cherché à les préparer par épimérisation des alcools 2 β , par l'intermédiaire des tosylates en solution dans le diméthylformamide. Mais les tosylates 7, 8 et 9 conduisant très rapidement à des produits d'élimination, de fragmentation ou de réarrangement (6,11) cette méthode s'est avérée négative.

Nous avons alors étudié les possibilités de réduction stéréosélective des oximes 16, 17 et 18, méthode classique d'introduction d'une fonction amine en position équatoriale dans un système cyclohexanique (6, 7, 8).

La réduction catalytique des oximes par l'oxyde de platine dans l'acide acétique, à pression ordinaire et température ambiante, de même que la réduction chimique par LiAlH_4 nous ont conduits à des mélanges d'épimères en quantités à peu près égales. Par contre, avec le sodium dans l'éthanol, nous avons obtenu un mélange où l'amino-2 β est très fortement majoritaire et facilement isolable sous forme

TABLEAU I

N°	R	Rdt %	F °C	Solvant de recristallisation	Éluant de chromato- graphie	IR cm^{-1}
7	H	76	146	benzène-éther isopropylique		1 600 aromatique disubstitué 1 170, 1 180 $\nu(\text{SO}_2)$
8	CH_3	85	154	benzène-éther isopropylique		1 595 aromatique disubstitué 1 170, 1 175 $\nu(\text{SO}_2)$
9	$\text{CH}_2 - \text{CH} \begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	77	142	éther isopropylique		1 592 aromatique disubstitué 1 172, 1 185 $\nu(\text{SO}_2)$
10	H	92	huile		benzène	2 100 N_3
11	CH_3	70	114		benzène-éther 25-75	2 100 N_3
12	$\text{CH}_2 - \text{CH} \begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	70	huile		benzène	2 090 N_3

TABLEAU II

N°	R	Rdt %	F °C	Solvant de recristallisation	IR cm^{-1}
Amino-2 α					
13	H	70	222-228	éthanol	3 420, 3 130 2 530 NH_3^+ 1 600, 1 570
14	CH_3	70	198-203	éthanol	3 380, 3 150 2 550 NH_3^+ 1 580
15	$-\text{CH}_2 - \text{CH} \begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	66	220-226	éthanol	3 400, 3 180 2 560 NH_3^+ 1 590, 1 565
Amino-2 β .					
19	H	70	211-216	éthanol	3 430, 3 430 2 060 NH_2^+ 1 640
20	CH_3	72	192-197	éthanol	3 480, 3 300 2 070 NH_3^+ 1 630
21	$-\text{CH}_2 - \text{CH} \begin{smallmatrix} \text{CH}_3 \\ \text{CH}_3 \end{smallmatrix}$	60	207-212	acétate d'éthyle éthanol	3 500, 3 400 2 050 NH_3^+

de chlorhydrate par cristallisation fractionnée. De cette façon, nous avons pu accéder aux amines 19, 20 et 21.

Ces amines épimères se différencient par leurs spectres infrarouge et leurs points de fusion. De plus, elles possèdent des polarités différentes en chromatographie sur couche mince ce qui permet de les identifier facilement.

Les caractéristiques physico-chimiques des composés nouveaux ont été rassemblées dans les tableaux I et II.

PARTIE EXPÉRIMENTALE.

Les points de fusion ont été déterminés sur un banc Kofler, à l'exception de ceux des chlorhydrates d'amines qui ont été pris à l'aide d'un microscope à platine chauffante.

Les spectres IR ont été enregistrés sur un spectrographe Perkin-Elmer modèle 257.

Toutes les réactions ont été suivies en chromatographie sur couche mince, exécutée sur Kieselgel-G selon STAHL. Les microanalyses ont été déterminées au Laboratoire de Chimie IV de la Faculté des Sciences d'Orléans.

Les alcools, les cétones et les oximes ont été préparés suivant des méthodes précédemment décrites (4, 9, 10).

Tosyloxy-2β méthyl-3α diméthoxy-9, 10 hexahydro-1,2,3,4, 6,7, 11b H benzo[a]quinolizine 8.

A une solution de 8,5 g de 5 (0,0306 mole) dans 70 cm³ de pyridine sèche, refroidie au bain de glace, sont ajoutés 17 g de chlorure de *para*-toluènesulfonyl fraîchement recristallisé. Après abandon à 0° pendant 20 h, le milieu réactionnel est versé dans l'eau glacée et alcalinisé à la lessive de soude. On essore 11,15 g de produit cherché. Rdt = 85 %, l'échantillon analytique est recristallisé dans un mélange benzène-éther isopropylique. F = 154°.

Analyse C₂₃H₂₉O₆NS = 431,53 : C, H, N.

Spectre IR (KBr) : 1 595 cm⁻¹ aromatique disubstitué 1 170-1 175 cm⁻¹, ν(SO₂).

Azido-2α méthyl-3α diméthoxy-9,10 hexahydro-1,2,3,4,6,7, 11b H benzo[a]quinolizine 11.

A une solution de 8 g de 8 (0,0185 mole) dans 120 cm³ de HMPT fraîchement distillé, sont ajoutés 16 g d'azoture de sodium. Après chauffage à 90° pendant 7 h, le mélange réactionnel est versé dans l'eau glacée et alcalinisé avec du carbonate de sodium. Après saturation de la phase aqueuse au chlorure de sodium et extraction au

benzène, l'évaporation du solvant laisse 5,5 g de résidu cristallin. Par chromatographie de 3,5 g de ce résidu sur alumine neutre, on isole dans les fractions benzène-éther (25-75) l'azide cherché qui, recristallisé dans l'éther, donne 2,5 g de cristaux blancs. Rdt = 70 %. F = 114°.

Analyse C₁₆H₂₂O₄N₄ = 302,37; C, H, N.

Spectre IR (nujol) : 2 100 cm⁻¹, — N₃.

Amino-2α méthyl-3α diméthoxy-9,10 hexahydro-1,2,3,4,6, 7, 11b H benzo[a]quinolizine 14.

Une solution de 1,88 g de 11 dans 35 cm³ d'alcool absolu est hydrogénée à pression atmosphérique et à température ambiante en présence de platine réduit pendant 10 h. La solution filtrée est neutralisée par 5,25 ml d'HCl 2 N, après évaporation du solvant, on obtient 1,6 g de produit cristallisé. Rdt = 70 %. L'échantillon analytique est recristallisé dans l'éthanol. F = 198-203°.

Analyse C₁₆H₂₆O₂N₂Cl₂, 1 H₂O = 367,28; C, H, N.

Spectre IR (KBr) : 3 380, 3 150, 2 550 cm⁻¹, NH₃⁺.

Amino-2β méthyl-3α diméthoxy-9,10 hexahydro-1,2,3,4, 6,7, 11b H benzo[a]quinolizine 20.

A une solution de 3 g de 17 dans 150 cm³ d'éthanol à reflux, sont ajoutés, par petites portions, 20 g de sodium. Vers la fin de l'addition, on ajoute 100 cm³ d'éthanol. La réaction étant terminée, le mélange réactionnel est versé dans l'eau glacée et l'amine formée est extraite au benzène. Après lavage avec une solution saturée de NaCl, la solution organique est évaporée à sec. Le résidu, repris dans l'éthanol est neutralisé par 8 ml d'HCl 2 N. Après évaporation du solvant et recristallisation dans l'éthanol, on obtient 2,6 g de produit pur cristallisé. Rdt = 72 %. F = 192-197°.

Analyse C₁₆H₂₆O₂N₂Cl₂, 1/4 H₂O = 353,79; C, H, N.

Spectre IR (KBr) : 3 480, 3 300, 2 070 cm⁻¹, NH₃⁺.

PARTIE PHARMACOLOGIQUE.

Les six amino-2 hexahydro benzo[a]quinolizines ont été étudiées à l'état de chlorhydrate.

Ces molécules se sont révélées comme pharmacologiquement actives :

- sur le système nerveux central, où elles exercent notamment une action sédative sur l'activité motrice et une action potentialisatrice de la narcose.
- sur le muscle lisse : *in vitro*, elles s'opposent au spasme produit par le chlorure de baryum; *in vivo*, chez le chien elles provoquent une vasodilatation.

TABEAU III

R =	N°	DL ₅₀ /kg I.V. souris	Activité motrice DE ₅₀ /kg I.V. souris	Potentialisa- tion narcose DE ₅₀ /kg I.V. souris	Augmentation du débit périphérique au 1/10 de la DE ₅₀ /kg I.V. chez le chien	Activité antispas- modique <i>in vitro</i> DE ₅₀
H	13 19	250 mg 300 mg	50 mg —	60 mg 40 mg	10 % —	— —
CH ₃	14 20	200 mg 150 mg	40 mg —	— —	15 % 10 %	4.10 ⁻⁵ 5.10 ⁻⁵
CH ₂ — CH	15	100 mg	20 mg	10 mg	60 %	3.10 ⁻⁵
	21	80 mg	—	32 mg	10 %	2.10 ⁻⁵

Ces activités restent modestes, l'amine la plus active (N° 15) est 8 fois moins sédatrice que la tétrabénazine et 10 fois moins antispasmodique que la papavérine prises comme étalons.

Cependant, leur étude comparative nous a permis de mettre en évidence deux types de constatations :

— l'allongement de la chaîne R accroît la toxicité et l'activité des molécules.

— l'épimérisation ne modifie pas la toxicité, mais les dérivés β ont pratiquement perdu l'activité manifestée par les dérivés α .

Le tableau III permet de comparer les résultats obtenus.

SUMMARY.

The two epimeres of 2-amino-9,10 dimethoxy-1,2,3,4,6,7, hexahydro 11b H benzo[a]quinolizines substituted or not in position-3 were prepared with a view to comparative pharmacological study. The α epimeres were obtained by a stereospecific method, the β epimeres by reduction of the corresponding oximes. These molecules show a low sedative, antispasmodic and vasodilating activity.

ZUSAMMENFASSUNG.

Die zwei Epimeren des 2-Amino-9,10-dimethoxy-1,2,3,4, 6,7, hexahydro 11b H benzo[a]chinolizin mit oder ohne

Substituenten in Stellung 3 wurden in Hinblick auf ihre pharmakologische Untersuchung dargestellt. Die α -Epimeren wurden mittels einer stereospezifischen Methode, die β -Epimeren durch Reduktion der entsprechenden Oxime erhalten. Diese Verbindungen besitzen eine schwache sedative, antispasmodische und gefässerweiternde Wirkung.

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Esters d'amino-alcools d'un acide dérivé du tropyliidène

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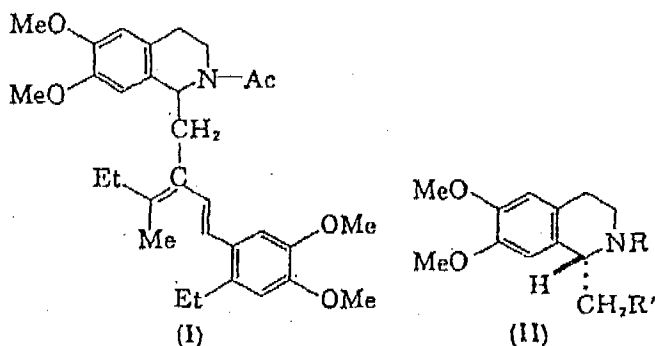
Le mémoire est consacré à l'étude d'une série d'esters d'amino-alcools de l'acide troyl-7 acétique. Leur synthèse et leur analyse spectroscopique ont été mentionnées, ainsi que les résultats d'une expérimentation pharmacodynamique préliminaire mettant en évidence une activité antispasmodique notable et une toxicité particulièrement faible pour certains de ces esters.

L'étude d'un acide dérivé du tropyliidène nous avait conduit à la préparation d'une série d'esters d'amino-alcools chez lesquels nous avons recherché des propriétés antispasmodiques (1). La structure de cet acide consistait en fait en une juxtaposition de cycles heptadiénique et propanique. Il était intéressant de comparer ces premiers dérivés (A) avec ceux de l'acide troyl-7 acétique (B) dans lequel le cycle propanique est supprimé.

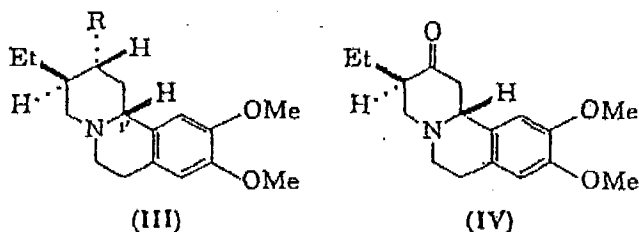
Nous décrivons ci-après la préparation de ces nouveaux esters (B), leurs caractéristiques ainsi que leur étude pharmacodynamique. L'acide troyl-acétique peut se préparer (2) à partir du bromure de tropyliidène (monobromé). Celui-ci, condensé avec l'acide malonique (3) donne l'acide troyl-malonique, puis, après décarboxylation, l'acide troylacétique dont le chlorure d'acide (4) réagit avec de bons rendements sur différents amino-

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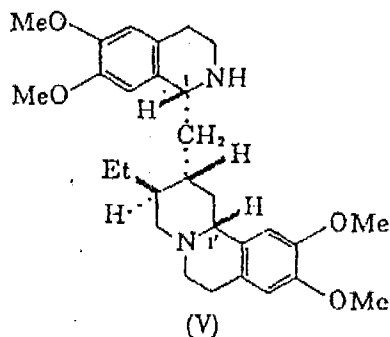
Stereochemistry of emetine. A. R. Battersby, R. Binks, D. Davidson, G. C. Davidson, and T. P. Edwards (Univ. Bristol, Engl.). *Chem. & Ind. (London)* 1957, 982; cf. *CA* 48, 10751d.—Hofmann degradation of *N*-acetylemetine repeated 3 times, with hydrogenation after the 1st step, gives I which, on oxidn. with O_3 and $AcOOH$, yields $MeEtCO$, 6-ethylveratric acid, and II [(R = Ac, R' = CO_2H), $[\alpha]_D -144^\circ$



(EtOH)]. The latter compd. is obtained by resolution of DL-tetrahydroisoquinoline, acetylation of the (+)-form, and hydrolysis. The (+)-form of II (R = H, R' = CO_2Et), and its 3 derivs., II (R = Et, R' = CH_2OAc , CH_2OH , Me), prepd. without affecting the asymmetric center, have rotations which are more neg. as the polarity of the solvent increases. *O*-methylpsychotrine, on oxidn. in alk. soln. and hydrogenolysis, gives III (R = CO_2H). The stereochemistry of the $COOH$ is based on the stability of the derived ester under vigorous equilibrating conditions; the C(1') configuration has been set arbitrarily to agree with that at



C(1) of emetine. Lack of inversion during the degradation is proved by Arndt-Eistert homologation of III (R = CO_2H) to III (R = CH_2CO_2H), which can be converted to *O*-methylpsychotrine. Curtius degradation of III (R = CO_2H) gives III (R = $NHCO_2Et$). IV with hydroxylamine acetate gives 2 major oximino derivs., which on redn. afford 4 isomeric amines. The Et urethan of the amine with the stereochemistry shown in IV is identical with that obtained by degradation of *O*-methylpsychotrine. Emetine is therefore V (abs. configuration at C(1') remains to be detd.).



C. L. Deasy

COMMUNICATIONS TO THE EDITOR

Contributions should have novelty or urgency. They should be brief, and submitted manuscripts, with Tables and Figures, should not exceed the equivalent of one printed page.

THE STEREOCHEMISTRY OF EMETINE

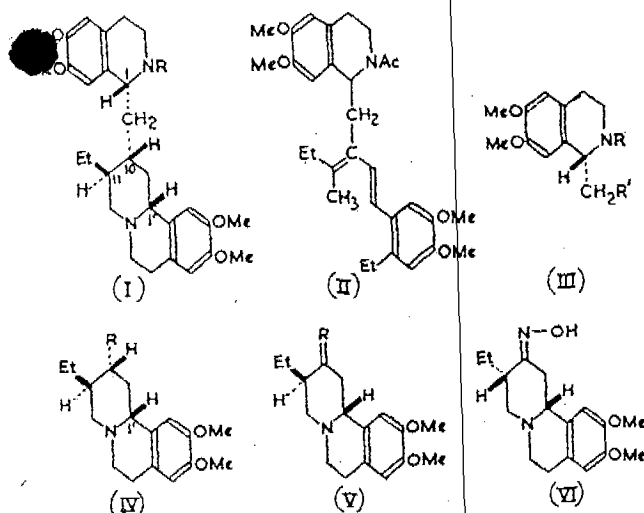
By A. R. Battersby, R. Binks, D. Davidson,
G. C. Davidson and T. P. Edwards

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The structure of emetine has been established by many degradative and synthetic investigations,^{1,3} but these did not reveal the stereochemistry of this important Ipecacuanha alkaloid. We report now our stereochemical studies on emetine.

Hofmann degradation of *N*-acetylemetine (I; R=H) was repeated three times, with hydrogenation after the first step,² to give the diene (II) which, when oxidized with ozone and peracetic acid, yielded methyl ethyl ketone, 6-ethylveratric acid and the acid (III; R=Ac, R'=CO₂H), [α]_D -144°(EtOH). The last was obtained in quantity by resolution of the synthetic³ DL-tetrahydroisoquinoline* (III; R=H, R'=CO₂Et), acetylation of the (+)-form and hydrolysis. The (+)-form of compound (III; R=H, R'=CO₂Et) was converted, by methods which do not affect the asymmetric centre, into three derivatives (III; R=Et, R'=CH₂OAc, CH₂OH, CH₃); these, and the parent compound (III; R=H; R'=CO₂Et), all have rotations which become more negative as the polarity of the solvent is increased. On this basis, the absolute configuration of (III) is as shown, and the absolute configuration at C(1) in emetine follows.

* Only one enantiomer is drawn of all DL-compounds in this and the following Communications.



The stereochemistry about the quinolizidine system in emetine is duplicated in *O*-methylpsychotrine⁴ (as (I), but lacking R and with double bond between C (1) and N). This alkaloid, as its bisbenzochloride, was oxidized in alkaline solution to a quaternary betaine from which the acid (IV; R=CO₂H) was obtained by hydrogenolysis. The equatorial conformation shown for the carboxyl group is based upon the stability of the derived ester under vigorous equilibrating conditions (in (IV), the absolute configuration at C (1') has been set arbitrarily the same as that at C (1) of emetine). That no inversion had occurred during the degradation was proved by Arndt-Eistert homologation of (IV; R=CO₂H) to (IV; R=CH₂CO₂H) which can be converted back into *O*-methylpsychotrine⁵; the stereochemistry at C (10) of emetine is therefore as shown. Curtius degradation of compound (IV; R=CO₂H) gave the urethane (IV; R=NH.CO₂Et).

The DL-ketone (V; R=O) had been synthesized earlier³ and equilibration studies now confirm that the ethyl group is equatorial. Treatment of this ketone with hydroxylamine acetate afforded two major oximino products, (V; R=N.OH) and (VI), together with two minor ones. Reduction of the former pair afforded four isomeric amines to which probable structures can be assigned by a conformational argument. The ethyl urethane of the amine assigned structure (IV; R=NH₂) had infrared absorption in solution identical with that of the urethane obtained above by degradation of *O*-methylpsychotrine. The *trans* arrangement at C (10), C (11) has been confirmed.⁶

The foregoing results lead to the stereochemistry of emetine as in (I; R=H) and only the absolute configuration at C (1') remains to be determined.

Received May 29, 1957

References

- 1 Späth & Pailer, *Monatsh.*, 1948, 78, 348; Battersby & Openshaw, *J. chem. Soc.*, 1949, 3207; Preobrashenski & his co-workers, *Doklady Akad. Nauk, S.S.S.R.*, 1950, 75, 539, and references cited in these papers
- 2 Cf. Pailer, *Monatsh.*, 1948, 79, 127
- 3 Battersby, Openshaw & Wood, *J. chem. Soc.*, 1953, 2463
- 4 Pyman, *ibid.*, 1917, 111, 415; Karrer, Eugster & Rüttner, *Helv. chim. Acta*, 1948, 31, 1219; Battersby & Davidson, forthcoming publication
- 5 Battersby, Davidson & Harper, following Communication
- 6 Battersby & Cox, following Communication