(8) H. J. Conn, *Stain Technol.*, **2**, 33(1927); *Ibid.*, **2**, 35(1927).
(9) J. Ball and D. S. Jackson, *Stain Technol.*, **28**, 33(1953).
(10) H. Budzikiewicz, C. Djerassi, and D. H. Williams, "Mass Spectrometry of Organic Compounds," Holden-Day, San Francisco, Calif., 1967, p. 23.
(11) E. Gurr, "Synthetic Dyes in Biology, Medicine and Chemistry," Academic, New York, N.Y., 1971, pp. 49, 58, 72.
(12) J. N. T. Gilbert and B. J. Millard, *Org. Mass Spectrom.*, **2**, 17(1969).
(13) S. Ohkuma and T. Furuhata, *Proc. Jap. Acad.*, **47**, 689(1971).
(14) K. Bergman and C. T. O'Konski, *J. Phys. Chem.*, **67**, 2169(1963).
(15) E. Rabinovitch and L. F. Epstein, *J. Amer. Chem. Soc.*, **63**, 69(1941).
(16) G. R. Haugen and E. R. Hardwick, *J. Phys. Chem.*, **67**, 725(1963).
(17) S. Kato, M. Morita, and M. Koizumi, *Bull. Chem. Soc. Jap.*, **37**, 117(1964).
(18) M. Koizumi, H. Obata, and S. Hayashi, *ibid.*, **37**, 108(1964).
(19) W. C. Holmes and E. F. Snyder, *Stain Technol.*, **4**, 7(1929).
(20) E. Gurr, "Synthetic Dyes in Biology, Medicine and Chemistry," Academic, New York, N.Y., 1971, p. 77.

ACKNOWLEDGMENTS AND ADDRESSES

Received September 3, 1974, from the *Department of Pharmaceutical Sciences, School of Pharmacy, University of Washington, Seattle, WA 98195*

Accepted for publication January 21, 1975.

Supported in part by the James Picker Foundation on the recommendation of the Committee on Radiology, National Academy of Sciences–National Research Council, and by National Institute of General Medical Sciences Grant GM 17271.

The authors thank Mr. Eliot Lieberman and Mr. William N. Howald for technical assistance and Dr. D. R. Allen, Dr. A. D. Blair, and Dr. A. L. Crittenden for advice and encouragement.

[x] To whom inquiries should be directed. Present address: Tumor Institute of the Swedish Hospital Medical Center, Division of Nuclear Medicine, Seattle, WA 98104

Inhibition of Synaptosomal Accumulation of *l*-Norepinephrine I: *N*-Arylalkyl and *N*-Aryloxyalkyl *dl*-Amphetamines and Related Compounds

JAMES C. SCHAEFFER, ARTHUR K. CHO [x], GLENN T. NAGAMI, and GLENN S. TAKIMOTO

Abstract □ The ability of a group of systematically modified amphetamines to inhibit the accumulation of *l*-norepinephrine by nonstriatal synaptosomes was investigated. *N*-Substitution by the proper bulky hydrophobic groups can be well tolerated. Structure–activity relationships generate a qualitative picture of the inhibitor–carrier interaction site.

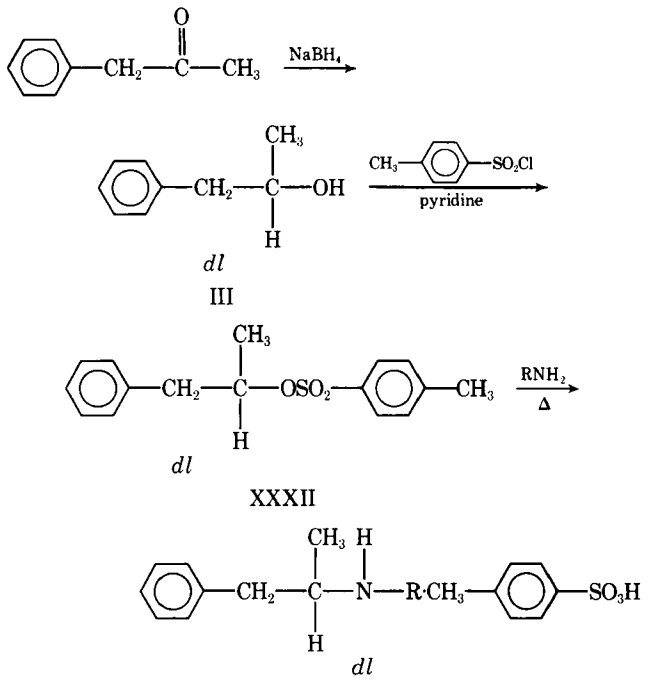
Keyphrases □ *l*-Norepinephrine accumulation by nonstriatal synaptosomes—inhibition by *N*-arylalkyl- and *N*-aryloxyalkyl-substituted amphetamines □ Amphetamines, *N*-substituted—inhibition of synaptosomal accumulation of *l*-norepinephrine □ Structure–activity relationships—*N*-substituted amphetamine inhibition of synaptosomal accumulation of *l*-norepinephrine

A catecholamine recycling mechanism located at the presynaptic neuronal membrane is thought to play a major role in the termination of noradrenergic nerve transmission and in the conservation of catecholamines (1–4). Antidepressant and stimulant drugs, such as desipramine (5), cocaine (6), and amphetamine (7), have been shown to inhibit this carrier-mediated transport process *in vitro*. Thus, at least part of their pharmacological action is believed to be derived from their ability to prolong effective catecholamine concentrations in the synaptic cleft and, therefore, at the postsynaptic receptors.

On the basis of kinetic (8) and differential inhibition (5, 9) data, it has been proposed that there are at least two different, anatomically separable, neuronal transport mechanisms for the catecholamines in the central nervous system (CNS). One is associated with the hypothalamus, cerebral cortex, midbrain, and medulla oblongata-pons, where *l*-norepinephrine is the major catecholamine present; the other is found in the corpus striatum, where dopamine is thought to be the important catecholamine transmitter. A wide range of tricyclic, sympathomimetic, anticholinergic, and antihistamine drugs (5, 9–11) have been shown to inhibit the *in vitro* uptake of catecholamines by both systems. However, few of these compounds exhibited a substantial preference for one of the transport processes. Even when selective inhibition was observed, as with desipramine, the less active nonstriatal process was usually the most susceptable to blockade.

In light of this evidence, a study was initiated to elucidate the differences and similarities of the two catecholamine uptake processes in the CNS by attempting to map the topography of the different carrier sites through the use of a series of amphetamine-related inhibitors. Structural features, which take advantage of the topographical variances thus uncovered, would then be integrated into the design of new inhibitors to increase specificity.

This report describes the effects of a group of amphetamine-related compounds on the uptake of *l*-norepinephrine by a synaptosome-enriched subcellular fraction from nonstriatal rat brain tissue. First,



Scheme I—Method A

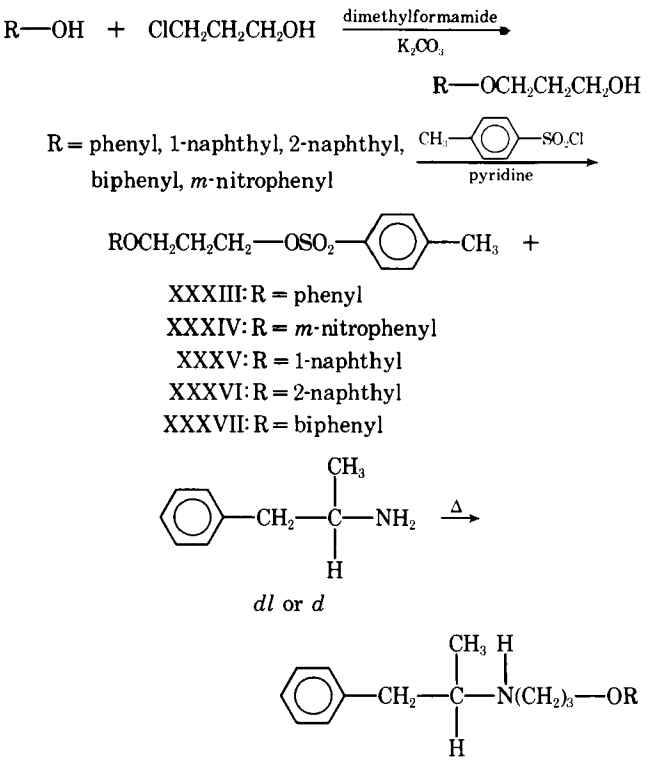
the relative inhibitor potency was investigated in a group of compounds whose structure was systematically modified by small increments to ascertain the important structural and physical–chemical properties connected with the ability of the transport system to recognize its substrate. This series also related the uptake system to previously published results.

Second, following the nonclassical inhibitor rationale of Baker and coworkers (12–14), a series of amphetamines substituted with bulky hydrophobic groups on the nitrogen was synthesized to study possible inhibitor interactions at sites on the carrier adjacent to the normal substrate binding position. The intrinsic affinity of the amphetamine moiety as a substrate mimic was utilized in these compounds to ensure potency, while the bulky substituents provided a means of enhancing selectivity through unique steric effects and/or hydrophobic interactions with adjacent sites on the carrier.

EXPERIMENTAL

Drugs—*S(d)*-Amphetamine[1] (IV), *R(l)*-amphetamine[1] (V), *dl*-amphetamine[1] (II), *dl*-tranylcypromine[1] (IX), *p*-hydroxy-*dl*-amphetamine[1] (VI), *m*-hydroxy-*dl*-amphetamine[1] (VII), phentermine[2] (VIII), *S(d)*-benzphetamine[3] (XVII), *dl*-prenylamine[4] (XXII), and ^{3}H-*l*-norepinephrine[5] (3–8 Ci/mmole) were obtained from commercial sources. The syntheses of other compounds are discussed.

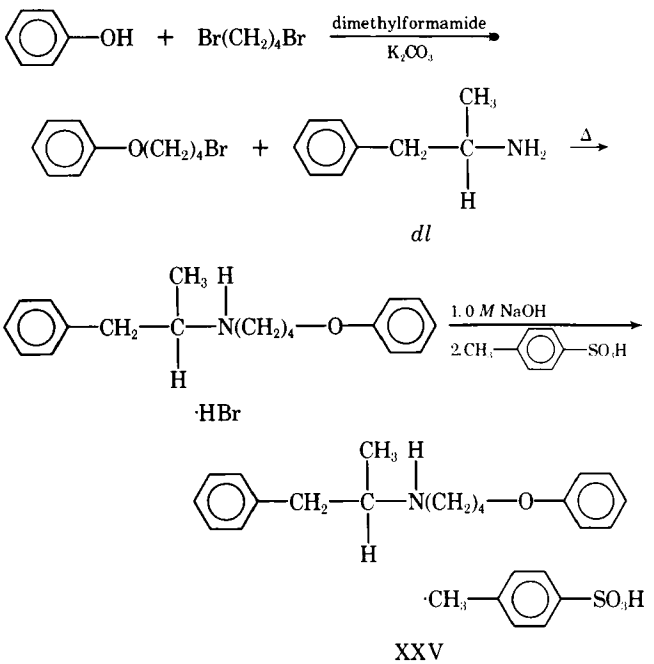
Chemical Methods—The key intermediate in Method A, α-methylphenethyl tosylate (XXXII), was prepared from phenylacetone as shown in Scheme I. This compound was synthesized previously from phenylacetaldehyde (15). Elevated temperatures (100°) and high reactant concentrations (neat conditions) were required to bring about satisfactory tosylate displacement by the



Scheme II—Method B

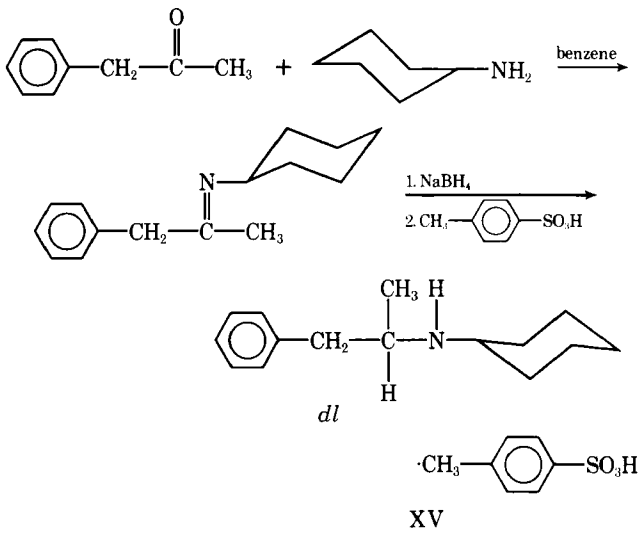
alkyl- or arylalkylamines employed. Equimolar amounts of tosylate (XXXII) and phenylethylamine in acetone at ambient temperature or reflux, or at 65–70° in dimethylformamide, gave only starting material. The reaction of aniline and XXXII at 100° yielded the aniline *p*-toluenesulfonate as the major product. Thus, elimination is apparently favored over substitution when arylamines are used. Compounds XIX–XXIII (Table I) were obtained *via* Method A.

Method B (Scheme II) was employed to synthesize XIV, XXIV, and XXVI–XXX (Table I). The physical properties of the 3-aryloxypropyl tosylate intermediates (XXXIII–XXXVII) are reported in Table II. Compound XXV was prepared from 4-phenoxybutyl bromide and *dl*-amphetamine according to Method C



Scheme III—Method C

[1] Smith Kline and French Laboratories, Philadelphia, Pa.
[2] Pennwalt Corp. Rochester, N.Y.
[3] The Upjohn Co., Kalamazoo, Mich.
[4] Segontin, Hoechst Pharmaceuticals, Somerville, N.J.
[5] New England Nuclear Corp., Boston, Mass.



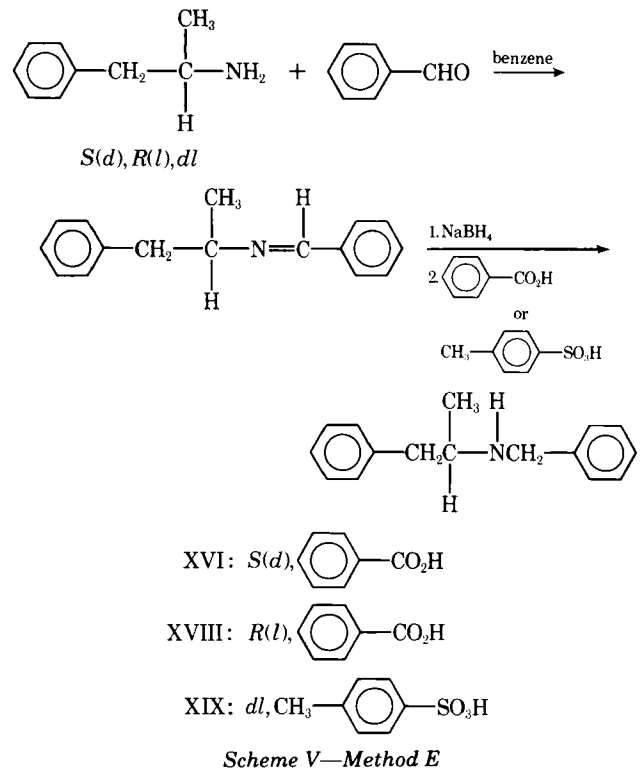
XV

Scheme IV—Method D

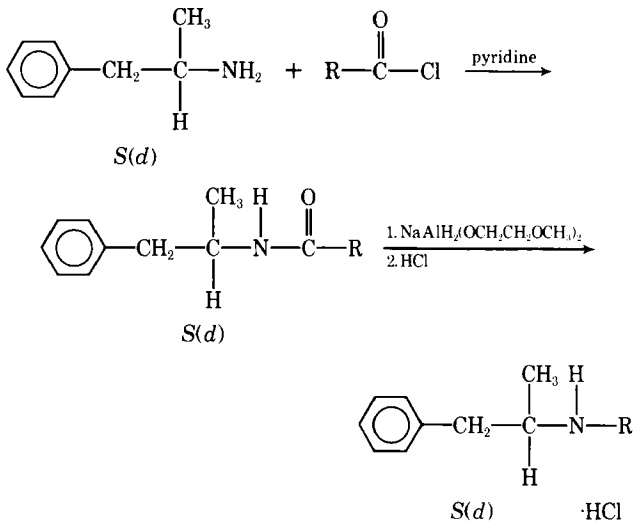
(Scheme III). The reaction of phenylacetone and cyclohexylamine, followed by sodium borohydride reduction, provided XV (Method D, Scheme IV).

To obtain the optical isomers of *N*-benzylamphetamine XVI and XVIII, Method E (Scheme V) was utilized. The benzoic acid salts of these compounds were much more easily recrystallized than the tosylates. The specific rotations of XVI and XVIII were +37.0 and −36.8°, respectively, at 24° (10% w/v, chloroform). The racemic compound, XIX, was also prepared by this method and was identical to that obtained *via* Method A. Method F (Scheme VI) was used to prepare XII and XIII.

Synthetic Procedures[6]—All melting points[7] reported here are uncorrected. The IR spectra were obtained using either neat liquids or mineral oil mulls[8]. At least one compound from each synthetic sequence involving an optically active center was checked on a polarimeter to ensure that the optical outcome of the synthesis was as expected.



Scheme V—Method E



XII: R = $-CH_2CH_3$

XIII: R = $-CH_2CH_2CH_3$

Scheme VI—Method F

General Procedure (Method A)—A mixture of 2 mmoles of *dl*-α-methylphenethyl tosylate (XXXII) and 2 mmoles of the appropriate arylalkylamine was heated at 95–100° for 1–2 hr. After the mixture had cooled to room temperature, the crude tosylate salt was recrystallized from the appropriate solvent and dried under high vacuum over phosphorus pentoxide at 60°. See Table I for additional data.

General Procedure (Method B)—A mixture of 0.1 mole of the appropriate phenolic compound, 0.1 mole of 3-chloro-1-hydroxypropane, 0.11 mole of anhydrous potassium carbonate, and 50 ml of dimethylformamide (dried over 4A molecular sieves) was heated to 85–90° for 18 hr. After the addition of 750 ml of water, the mixture was extracted twice with 150 ml of chloroform. These extracts were combined, backwashed three times with 100 ml of 1 *N* NaOH and three times with 100 ml of water, and dried with magnesium sulfate. The solvent was removed by spin evaporation to give the crude 3-aryloxypropanol. These materials were not rigorously characterized but were sufficiently pure to be used in the next step.

To a solution of 10 mmoles of the appropriate 3-aryloxypropanol in 20 ml of pyridine (dried over potassium hydroxide pellets) was added 2.87 g of freshly recrystallized *p*-toluenesulfonyl chloride (15 mmoles). This mixture was allowed to stand in a refrigerator at 5° for 2 days, during which time pyridine hydrochloride crystallized out. After 400 ml of water was added to the reaction mixture, it was extracted with three 50-ml portions of ether. The ether extract was backwashed with 1 *N* HCl and water, dried with magnesium sulfate, and spin evaporated to the crude tosylate, which was recrystallized from the solvent listed in Table II.

An equimolar mixture of *dl*- or *d*-amphetamine and the appropriate 3-aryloxypropyl tosylate was treated as described in Method A to yield the corresponding *N*-(3-aryloxypropyl)amphetamine. See Table I for further information.

*N-(4-Phenoxybutyl)-*dl*-amphetamine p-Toluenesulfonate (XXV) (Method C)*—A mixture of 9.4 g of phenol (0.1 mole), 100 g of 1,4-dibromobutane (0.46 mole), 15.2 g of potassium carbonate, and 50 ml of dimethylformamide (dried over 4A molecular sieves) was heated at 65° for 6 hr. After the addition of 300 ml of water, the mixture was extracted with 300 ml of heptane. This extract was backwashed once with 100 ml of 1 *N* NaOH and three times with 100 ml of water, dried with magnesium sulfate, and spin evaporated to an oil. This crude mixture was fractionated by high vacuum distillation. The fraction distilling between 113 and 115°/0.5 mm was collected and shown to be 4-phenoxybutyl bromide (5.2 g, 22%) of sufficient purity for use in the next step.

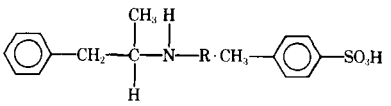
Two hundred and seventy milligrams of *dl*-amphetamine (2

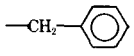
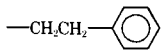
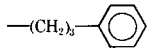
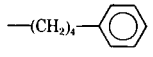
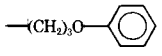
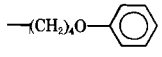
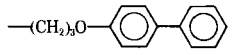
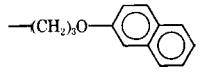
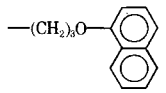
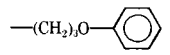
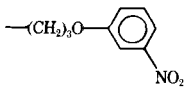
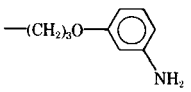
[6] Elemental analyses were performed by H. King, Department of Chemistry, University of California at Los Angeles.

[7] Mel-Temp apparatus.

[8] Infracord, Perkin-Elmer Corp.

Table I—*N*-Alkyl, Arylalkyl, and Aryloxyalkyl *dl*-Amphetamine *p*-Toluenesulfonates[a]



Compound	R	Method	Recrystallization Solvent	Yield, %[b]	Melting Point
XII[c,d,e]	$-CH_2CH_3$	F	Ethanol–acetone (1:5)	10	153–155°
XIII[d,e]	$-CH_2CH_2CH_3$	F	Ethanol	12	183–184°
XIV[f]	$-CH_2CH_2CH_2CH_3$	B[g]	Ether–acetone (5:1)	27	119–121°
XV		D	Ether–acetone (5:1)	29	133–135°
XIX[h]		A, E	Acetone	20	158–161°
XX		A	Acetone	12	160–161°
XXI[i]		A	Acetone	23	156–158°
XXIII		A	Acetone	28	130–132°
XXIV		B	Ethanol	45	164–166°
XXV		C	Acetone	17	132–134°
XXVI		B	Ethanol–acetone (1:2)	39	98–101°
XXVII		B	Acetone–ether (1:2)	26	141–143°
XXVIII		B	Acetone	41	135–137°
XXIX[d]		B	Ethanol–acetone	68	167–169°
XXX[d]		B	Acetone	37	127–130°
XXXI[d,j]		—	Ethanol	40	152–155°

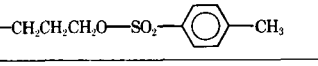
[a] All compounds were uniform by TLC (free base, methanol) and gave satisfactory carbon and hydrogen analyses. [b] Minimum recrystallized yields from last step in synthetic sequence. [c] N. J. Leonard, J. A. Adamick, C. Djerassi, and O. Halpern, *J. Amer. Chem. Soc.*, **80**, 4858 (1958). Apetinil, mp 154–156°. [d] *S(d)*-Isomer. [e] Hydrochloride salt. [f] Hydrochloride salt previously prepared by M. Friefelder and G. R. Stone, U.S. pat. 3,014,966 (1961). [g] Prepared by heating equimolar amounts of *dl*-amphetamine and butyl tosylate at 90–95° for 3 hr. [h] Hydrochloride salt previously prepared by E. H. Woodruff, J. P. Lambody, and W. E. Burt, *J. Amer. Chem. Soc.*, **62**, 922(1940). [i] Hydrochloride salt previously prepared by E. Bumm, British pat. 700,722 (1953). [j] Di-*p*-toluenesulfonate salt. Prepared from the corresponding nitro compound.

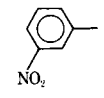
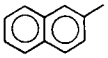
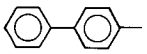
mmoles) and 458 mg of 4-phenoxybutyl bromide (2 mmoles) were heated at 95° for 3 hr. After the crude reaction product was cooled to ambient temperature, 25 ml of 1.0 *N* NaOH was added. This mixture was extracted twice with 25 ml of chloroform, and the extract was dried with magnesium sulfate and spin evaporated to an oil. The oil was dissolved in 8 ml of acetone, and a small amount of insolubles was removed by filtration. Then 380 mg of *p*-toluenesulfonic acid hydrate (2 mmoles) was added to the filtrate. After this mixture stood overnight in a refrigerator, a crystalline precipitate formed. This material was filtered, recrystallized twice from acetone, and pumped dry under high vacuum over phosphorus pentoxide. See Table I for additional data.

N-*Cyclohexyl*-dl-*amphetamine* p-*Toluenesulfonate (XV) (Method D)*—A mixture of 6.7 g of phenylacetone (0.05 mole), 5.54 g of cyclohexylamine (0.055 mole), and 100 ml of benzene was distilled until the vapor temperature of the distillate reached 80°. Benzene was added as necessary. After the reaction had gone to completion, the remaining benzene was removed by spin evaporation. To the residual oil were added 100 ml of ethanol and 6.27 g of sodium borohydride (165 mmoles). After the mixture was refluxed for 4 hr, the ethanol was removed under vacuum and 250 ml of 2 *M* NaOH was added to the residue. This mixture was extracted with 150 ml of ether. The extract was dried with magnesium sulfate and spin evaporated to an oil, which was distilled under high vacuum. The fraction distilling at 111–112°/0.3 mm was collected and shown to be *N*-cyclohexyl-*dl*-amphetamine (3.1 g, 29%). It was converted to its analytically pure tosylate salt by treatment with *p*-toluenesulfonic acid hydrate. See Table I for additional information.

General Procedure (Method E)—The benzene–water azeotrope was distilled from a mixture of 6.75 g of the appropriate amphetamine (50 mmoles), 5.83 g of benzaldehyde, and 100 ml of benzene

Table II—3-Aryloxypropyl Tosylates[a]



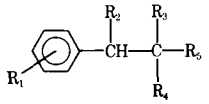
Compound	R	Method	Recrystallization Solvent	Yield, %[b]	Melting Point
XXXIII		B	Ether–hexane (1:1)	57	48–51°
XXXIV		B	Chloroform–ether (1:10)	63	74–75°
XXXV		B	Ether	56	69–71°
XXXVI		B	Ether	55	67–68°
XXXVII		B	Ether	52	96–98°

[a] All compounds moved as single spots on TLC [chloroform–hexane (1:1)] and gave satisfactory carbon and hydrogen analyses. [b] Minimum recrystallized yields.

until the vapor temperature of the distillate reached that of pure benzene. Benzene was added as necessary to maintain a reasonable reaction mixture volume. After the remaining benzene was removed under vacuum, 100 ml of ethanol and 6.27 g of sodium borohydride (165 mmoles) were added. Following a 4-hr reflux, the mixture was spin evaporated to a residue and 250 ml of 2 *M* NaOH was added. The resulting mixture was extracted with 150 ml of ether. The extract was dried with magnesium sulfate and spin evaporated to an oil, which was vacuum distilled.

*N-Benzyl-*d*-amphetamine and N-Benzyl-*l*-amphetamine Benzoates (XVI and XVIII)*—The fraction distilling at 128–130°/0.2 mm was collected (yield of 35–40%). To a solution of 900 mg of either amphetamine isomer (4 mmoles) in 10 ml of acetone was added 488 mg of benzoic acid (4 mmoles). After the solution was cooled overnight, a crystalline precipitate formed; this precipitate was recrystallized from acetone and pumped dry under vacuum over phosphorus pentoxide at 60°. The free bases of both isomers exhibited the same IR spectra, and the benzoate salts both melted at 101–103°. A 10% (w/v) chloroform solution of the free base of the *S(d)*-isomer had a specific rotation of +37.0°, whereas the *R(l)*-isomer had a specific rotation of −36.8°. An elemental analysis of the *R(l)*-isomer gave the following data. Calc. for C, 79.50; H, 7.25. Found: C, 79.29; H, 7.11.

Table III—Inhibition of the Synaptosomal Uptake[a] of *l*-Norepinephrine by Compounds I–IX



Compound	R_1	R_2	R_3	R_4	R_5	Optical Isomer	I_{50}, μM[b]
I	H	H	H	H	NH_2	—	6.0
II	H	H	CH_3	H	NH_2	*dl*	1.3
III[c]	H	H	CH_3	H	OH	*dl*	>>100[d]
IV	H	H	CH_3	H	NH_2	*S(d)*	0.9
V	H	H	CH_3	H	NH_2	*R(l)*	2.3
VI	*p*-OH	H	CH_3	H	NH_2	*dl*	1.1
VII	*m*-OH	H	CH_3	H	NH_2	*dl*	1.8
VIII	H	H	CH_3	CH_3	NH_2	—	1.6
IX	H	—CH_2—		H	NH_2	*dl*	3.7

[a] Substrate concentration was 1 μM *l*-norepinephrine. [b] All values are the average of two or more independent determinations. The variation in each multiple determination was less than 25%. [c] C. Golumbia and D. L. Cottle, *J. Amer. Chem. Soc.*, 61, 996(1939). [d] No inhibition at 100 μM.

*N-Benzyl-*dl*-amphetamine p-Toluenesulfonate (XIX)*—The fraction distilling at 136–137°/0.2 mm was collected (yield of 52%). To a solution of 450 mg of *N*-benzyl-*dl*-amphetamine (2 mmoles) in 40 ml of acetone was added 380 mg of *p*-toluenesulfonic acid hydrate (2 mmoles). After the mixture stood at room temperature overnight, a solid had precipitated. It was filtered, recrystallized from acetone, and dried under high vacuum over phosphorus pentoxide at 60°. The free base of this material did not rotate polarized light. The *p*-toluenesulfonate was identical to that prepared by Method A.

General Procedure (Method F)—To a solution of 6.75 g of *d*-amphetamine (50 mmoles) and 50 ml of pyridine (dried over potassium hydroxide) in an ice bath was added slowly 50 mmoles of the appropriate acid chloride. After this mixture was allowed to stir at room temperature for 25 hr, 750 ml of water was added and the resulting mixture was extracted with three 100-ml portions of chloroform. The extract was backwashed three times with 100 ml of 1 *M* HCl, three times with 100 ml of 2 *M* NaOH, and three times with 100 ml of water; it was then dried with magnesium sulfate and spin evaporated to a residue. The crude amide was dissolved in 50 ml of benzene, and 11.5 g of sodium bis(2-methoxyethoxy) aluminum hydride[9] (40 mmoles) dissolved in 50 ml of benzene was added.

This mixture was refluxed for 27 hr and cooled to 0–4°, and then 300 ml of 1 *N* NaOH was added slowly followed by 100 ml of benzene. The benzene layer was separated, dried with magnesium sulfate, and spin evaporated to an oil. The oil was dissolved in 100 ml of ether, and dry hydrogen chloride gas was bubbled through the solution. The resulting precipitate was filtered, recrystallized, and dried. Further information about XII and XIII, prepared by this method, is presented in Table I.

*N-[3-(3-Aminophenoxy)propyl]-*d*-amphetamine (XXXI)*—A mixture of 974 mg of *N*-[3-(3-nitrophenoxy)propyl]-*d*-amphetamine *p*-toluenesulfonate (XXX) (2 mmoles), 40 ml of ethanol, 5 ml of acetic acid, 5 ml of water, and 1.31 g of zinc powder (20 mmoles) was refluxed for 18 hr. The supernate was decanted from the zinc powder and spin evaporated to a residue. After 150 ml of 1 *N* NaOH was added to the residue, this mixture was extracted with three 50-ml portions of benzene. The extract was dried with magnesium sulfate and evaporated under reduced pressure to an oil. This material was dissolved in 20 ml of ethanol, which contained 760 mg of *p*-toluenesulfonic acid hydrate (4 mmoles). After this solution was cooled, a solid precipitated, which was recrystallized from ethanol and dried under high vacuum over phosphorus pentoxide at 60°. Physical data are provided in Table I.

Filtration Assay Method—*P_2' Preparation*—Male Sprague–Dawley rats, 180–200 g, were killed by decapitation. The brains were immediately removed, and the cerebellum and corpus striatum of each were rapidly dissected and discarded. The remaining brain tissue, henceforth referred to as nonstriatal tissue (1.2 g/brain), was homogenized in nine volumes of ice-cold 0.32 *M* sucrose, using a glass homogenizer with a polytetrafluoroethylene pestle. All subsequent procedures were carried out in ice-cold equipment or at 0–4° in a refrigerated centrifuge[10].

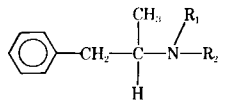
By employing essentially the procedure of Gray and Whittaker (16), a P_2' fraction enriched with synaptosomes was obtained as follows. The brain homogenate was centrifuged for 10 min at 1000×*g* to sediment debris and nuclei. The supernate was carefully separated and centrifuged at 11,000×*g* for 20 min. The resulting pellet (P_2) was uniformly resuspended by hand in 10 ml of 0.32 *M* sucrose/1.2 g of original brain tissue. This suspension was centrifuged for 20 min at 11,000×*g*. The supernate was discarded and the pellet (P_2' or P_2 washed) was resuspended in 2 ml of 0.32 *M* sucrose/1.2 g of original brain weight to give a preparation containing 15–20 mg of protein/ml. All P_2' fractions were used immediately after preparation.

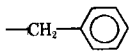
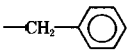
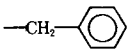
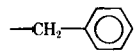
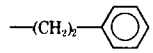
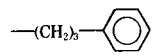
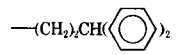
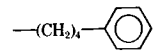
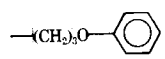
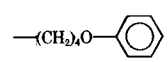
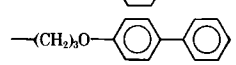
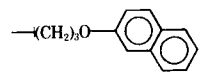
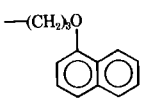
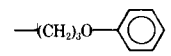
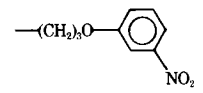
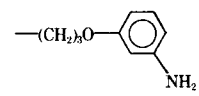
Protein concentrations were measured by the procedure of Lowry *et al.* (17), using bovine serum albumin as the standard.

Assay Procedure—A mixture of 0.53 ml of Krebs–Ringer phosphate buffer (pH 7.4) containing *l*-ascorbate, glucose, and edetic acid, 0.02 ml of 0.19 m*M* nialamide, 0.05 ml of 0.1 *M* NaH_2PO_4–

[9] Red-Al, Aldrich Chemical Co.
[10] Sorvall RC-2B, Dupont Instruments.

Table IV—Inhibition of the Synaptosomal Uptake[a] of *l*-Norepinephrine by Compounds X–XXXI



Compound	R_1	R_2	Optical Isomer	I_{50}, μM[b]	I_{50}, μM[c] (Ionized)
X	H	CH_3	*S(d)*	1.2	1.2
XI[d]	CH_3	CH_3	*S(d)*	7.5	7.5
XII	H	$-CH_2CH_3$	*S(d)*	2.3	2.3
XIII	H	$-(CH_2)_2CH_3$	*S(d)*	8.0	8.0
XIV	H	$-(CH_2)_3CH_3$	*dl*	12.3	12.3
XV	H		*dl*	10.5	10.5
XVI	H		*S(d)*	11.3	6.3
XVII	CH_3		*S(d)*	29.8	3.7
XVIII	H		*R(l)*	4.4	2.5
XIX	H		*dl*	5.0	2.8
XX	H		*dl*	7.3	7.3
XXI	H		*dl*	4.4	4.4
XXII	H		*dl*	5.7	5.7
XXIII	H		*dl*	2.7	2.7
XXIV	H		*dl*	4.4	4.4
XXV	H		*dl*	2.1	2.1
XXVI	H		*dl*	13.3	13.3
XXVII	H		*dl*	5.8	5.8
XXVIII	H		*dl*	5.2	5.2
XXIX	H		*S(d)*	3.7	3.7
XXX	H		*S(d)*	3.8	3.8
XXXI	H		*S(d)*	2.1	2.1

[a] Substrate concentration was 1 μM *l*-norepinephrine. [b] All values are the average of two or more independent determinations. The variation in each multiple measurement was less than 25%. [c] The I_{50} values listed in this column are corrected for the concentration of the cationic species at pH 7.4. All compounds, except for XVI–XIX, have a pKa of 9.5–10.0; therefore, less than 1% is nonionized under normal assay conditions. [d] Prepared by the Eschweiler–Clarke methylation of *d*-amphetamine. S. Senoh and I. Mita, *J. Pharm. Soc. Jap.*, 72, 1096(1952).

Na_2HPO_4 buffer (pH 7.4) with or without inhibitor, and 0.10 ml of the P_2' preparation was preincubated on a metabolic shaker at 37°. After 5 min, 0.05 ml of 15 μM ^{3}H-*l*-norepinephrine was added to give a 1-μM final concentration. The final concentrations of other components of this incubation medium were 123 mM Na^+, 4.2 mM K^+, 2.5 mM Mg^{+2}, 0.9 mM Ca^{+2}, 8.4 mM *d*-glucose, 1.1 mM *l*-ascorbate, 0.064 mM edetic acid, 42.6 mM sucrose, and 12.5 μM nialamide.

After incubation at 37° for 5 min in the presence of 1 μM substrate, the reaction was quenched by the addition of 5 ml of ice-cold 0.9% NaCl solution, which contained 100 μM unlabeled *l*-norepinephrine. This mixture was rapidly filtered through a 0.8-μm cellulose acetate filter[11] (18). The filter was then washed twice with 5-ml aliquots of quench, dissolved in 10 ml of Bray's counting solution (19) by shaking for 30 min, and counted[12]. Blanks for all inhibitor concentrations were obtained by repeating this procedure for 1 min at 0°.

All points were done in triplicate, and the mean value was used to determine picomoles of *l*-norepinephrine uptake in the fol-

[11] Millipore Corp., Bedford, Mass.
[12] All counting was done on an Amersham-Searle Mark II liquid scintillation counter.

lowing manner. The net counts accumulated for each experimental point, $CPM_{37}-CPM_0$ (CPM_{37} represents the radioactivity accumulated at 37°, and CPM_0 represents the radioactivity accumulated at 0°), were divided by the specific activity (counts per minute per picomole) of the ^3H-*l*-norepinephrine stock solution used for each experiment. The specific activity was determined by counting a mixture of 0.05 ml of the radioactive stock solution, 10 ml of Bray's, and a cellulose acetate filter that had gone through the normal incubation and filtration procedure without [^3H]-*l*-norepinephrine. Quenching was shown to be uniform for all samples in each experiment by the standard channels ratio method.

I_{50} Determinations (20)—The accumulation of 1 μM ^3H-*l*-norepinephrine was determined in the presence of three different inhibitor concentrations that reduced control uptake by 30–70%. The ratio of A_0/A_I, where A_0 represents control accumulation (picomoles per milligram of protein) and A_I represents accumulation with inhibitor, was plotted against [I]. These points were fitted by the method of least squares to give the best line. The point at which $A_0/A_I = 2$ provided the I_{50} values.

RESULTS AND DISCUSSION

The compounds in Table III were used as probes to determine the important substrate–carrier interaction points by measuring the relative potency of their inhibitory power. The simple phenethylamine (I) skeleton of *l*-norepinephrine was a good inhibitor. Introduction of an α-methyl group, the conversion of I to *dl*-amphetamine (II), caused a fivefold increase in inhibitor strength. Similar observations have been made using the perfused rat heart (21) and using a crude synaptosomal preparation (S_1) from rat brain hypothalamus (10). The presence of a monoamine oxidase inhibitor (nialamide) in the incubation mixture ruled out the possibility of this result being more a reflection of metabolism than enhanced inhibitor–carrier association. Thus, the introduction of a small hydrophobic substituent at the α-position has a positive effect. Phentermine (VIII) was as good an inhibitor as *dl*-amphetamine, so that addition of a second α-methyl group can also be tolerated. The inclusion of the α-methyl group of amphetamine in a cyclopropyl ring (tranylcypromine, IX), on the other hand, reduced carrier interaction.

The replacement of the basic and polar amine function of II with a neutral and polar hydroxy group (III) led to complete loss of inhibition. This finding indicates the critical role of the positively charged amine function in substrate recognition and appears to rule out the possibility of a strong interaction through hydrogen bond formation. The carrier molecule appears to contain a negatively charged group at its substrate combining site.

Consistent with recently reported results (22, 23), the *S(d)*-isomer of amphetamine (IV) was twice as potent as the *R(l)*-enantiomer (V). Coyle and Synder (7) previously reported a 10-fold difference in the inhibitor potency of these compounds, but they (7, 9) pretreated their animals with 5–2.5 mg/kg of reserpine to deplete catecholamines and inactivate the vesicular storage mechanism so the tissue sources were different.

The substitution of hydroxy groups on the benzene ring (VI and VII) did not alter inhibitor potency to a great extent, so the phenolic hydroxys of *l*-norepinephrine do not appear to play a major role in substrate–carrier binding. The only case in which ring hydroxylation substantially increases inhibition potency is that of tyramine compared to phenylethylamine (10, 11). However, this result may also reflect the well-known releasing effect of tyramine (23). The general structural feature of the substrate required for carrier binding is the phenethylamine structure. The introduction of one or two α-methyl groups enhances inhibitor strength and if one α-methyl is present, the *S(d)*-optical configuration is optimal.

Table IV summarizes investigations into the effect of *N*-substitution on uptake inhibitor strength to see whether large groups at this position could be tolerated by the carrier. Methamphetamine (X) was essentially as good an inhibitor as *S(d)*-amphetamine (IV). However, the addition of a second methyl group (XI) did result in a sixfold loss in inhibitor strength. There are other examples of the drop in potency with increasing *N*-substitution. Inhibition of *l*-norepinephrine uptake into hypothalamic preparations by desipramine is likewise reduced when the compound is converted to imipramine (5). Furthermore, it is well known that successive methylation of amphetamine causes a marked loss of pressor activity (24). The anionic carrier site does not appear to be able to accommodate a tertiary amine as well as a secondary amine, probably due to increased steric hindrance caused by the third ligand.

Since a secondary amine structure can be tolerated, a homologous series of *N*-alkyl amphetamines (XII–XIV) was investigated. Each successive lengthening of the alkyl substituent by one carbon resulted in a corresponding decrease in binding. The bulky cyclohexyl substituent of XV was no worse than the butyl substituent of XIV in decreasing effective carrier interaction. This finding suggests that only one edge of the ring interacts with the carrier surface. Again, steric factors are probably responsible for the observed reductions in inhibitor strength, because there is no correlation between increased molecular hydrophobicity and inhibitor potency.

The addition of a phenyl ring to methamphetamine (XVI) precipitates an apparent 10-fold loss in effectiveness, which could be the result of unfavorable steric requirements. However, the pKa of *N*-benzylamphetamine is 7.5 (25); thus 44% of this compound is present as the neutral form under the assay conditions employed (pH 7.4) in contrast to methamphetamine, which is 99% ionized at pH 7.4. Since a protonated amine is required for strong carrier interaction (II *versus* III), the substantial decrease in inhibitory power could be partially the result of the decreased concentration of the ionized form. Indeed, after the I_{50} value was corrected for ionization, only a fivefold loss could be attributed to steric factors. Similarly, the corrected I_{50} value for benzphetamine (XVII) cation (pKa = 6.55) (25) is 3.7 μM. Thus, in this case, the methylation of a secondary amine does not greatly change the potency of the ionized form (XVI *versus* XVII).

N-Benzyl substitution also reversed the relative inhibitory potency of the optical isomers. The *R(l)*-isomer (XVIII) was at least twice as potent as the *S(d)*-isomer in blocking *l*-norepinephrine accumulation. The sevenfold loss (corrected I_{50} values) in inhibitory power associated with the conversion of *S(d)*-amphetamine (IV) to *N*-benzyl-*S(d)*-amphetamine (XVI) is reduced to almost none for the corresponding *R(l)*-isomers (V and XVIII). The mode of interaction of the *R(l)*-isomer apparently allows greater steric toleration of the bulky benzyl group or may bring this hydrophobic function into contact with a hydrophobic patch on the transport carrier. The racemic *N*-benzyl-*dl*-amphetamine (XIX) had an intermediate I_{50} value as expected and had half the potency of *dl*-amphetamine (II).

N-Phenethyl-*dl*-amphetamine (XX) was three to four times less potent than *N*-ethyl-*d*-amphetamine (XII). Since the pKa values of these compounds are similar, this loss in inhibitor efficiency can probably be best attributed to steric hindrance. A comparison of the *N*-benzyl (XIX) and *N*-phenethyl (XX) compounds shows that the introduction of a second methylene group causes a twofold decrease in binding after correction for pKa differences. With the substitution of a *N*-phenylpropyl group (XXI), a twofold increase in potency was observed over the simple *N*-propyl derivative. This compound also exhibited twofold greater potency than the phenethyl homolog. Increased chain length and flexibility have apparently brought the phenyl ring into position where positive hydrophobic forces come into operation. The addition of a second phenyl substituent (XXII) slightly decreased inhibitor strength.

The placement of a butyl chain between the terminal phenyl ring and the nitrogen of *dl*-amphetamine (XXIII) slightly decreased the I_{50} value when compared to the corresponding propyl derivative (XXI). This compound was five times as potent as *N*-butyl-*dl*-amphetamine (XIV). Once again, strong hydrophobic interaction of the phenyl ring with the carrier site is the best explanation. Neither the substitution of an ether oxygen for a methylene (XXIV) nor the lengthening of the chain to five atoms (XXV) had much effect on inhibitor strength.

The next series of compounds was synthesized and tested to investigate the dimensions and properties of the proposed hydrophobic site adjacent to the normal substrate-combining region on the *l*-norepinephrine carrier. The substitution of a *p*-phenyl ring on XXIV to form the biphenyl derivative (XXVI) resulted in a fourfold loss in binding. The fusion of an aromatic ring to XXIV at the *meta/para*-position (XXVII) or *meta/ortho*-position (XXVIII) gave inhibitors that were only slightly less effective. Thus, the hydrophobic carrier site can tolerate the planar 2-naphthyl (XXVII) or 1-naphthyl (XXVIII) substituent, whereas a large planar biphenyl appears to exceed the dimensions of the hydrophobic pocket.

The possibility that electronic forces play a significant role in

the interaction of the terminal phenyl ring of the *N*-substituent can be ruled out by XXX and XXXI. The substitution of either a strong electron-withdrawing nitro group (XXX) or a strong electron-donating amino group (XXXI) resulted in only minor fluctuations in inhibitor strength. Additionally, the unaltered inhibitor potency after the addition of a polar amino group to XXIV indicates that only part of the terminal phenyl ring is involved in a hydrophobic interaction with the carrier molecule.

Overall, there appears to be no simple correlation between hydrophobicity and inhibitor strength in this series of compounds as has been reported for primary alcohols (26) and certain psychotropic drugs (27). The introduction of the proper bulky groups on the amphetamine nitrogen can be tolerated with little loss in inhibitor potency. Thus, the preparation of specific nonclassical inhibitors with substitution at the nitrogen seems possible.

REFERENCES

(1) J. Axelrod, *Recent Prog. Horm. Res.*, **21**, 597(1965).

(2) L. L. Iversen, "The Uptake and Storage of Noradrenaline in Sympathetic Nerves," Cambridge University Press, New York, N.Y., 1967.

(3) N. E. Anden, A. Carlsson, and J. Haggendal, *Ann. Rev. Pharmacol.*, **9**, 119(1969).

(4) L. L. Iversen, *Brit. Med. Bull.*, **29**, 130(1973).

(5) A. S. Horn, J. T. Coyle, and S. H. Snyder, *Mol. Pharmacol.*, **7**, 66(1971).

(6) S. B. Ross and A. L. Renyi, *Eur. J. Pharmacol.*, **2**, 181(1967).

(7) J. T. Coyle and S. H. Snyder, *J. Pharmacol. Exp. Ther.*, **170**, 221(1969).

(8) *Ibid.*, **165**, 78(1969).

(9) J. T. Coyle and S. H. Snyder, *Science*, **166**, 899(1969).

(10) A. S. Horn and S. H. Snyder, *J. Pharmacol. Exp. Ther.*, **180**, 523(1972).

(11) A. S. Horn, *Brit. J. Pharmacol.*, **47**, 332(1973).

(12) B. R. Baker, *Cancer Chemother. Rep.*, **4**, 1(1959).

(13) B. R. Baker, W. W. Lee, W. A. Skinner, A. P. Martinez, and E. Tong, *J. Med. Pharm. Chem.*, **2**, 633(1960).

(14) B. R. Baker, "Design of Active-Site-Directed Irreversible Enzyme Inhibitors," Wiley, New York, N.Y., 1967.

(15) B. Lindeke and A. K. Cho, *Acta Pharm. Suecica*, **9**, 363 (1972).

(16) E. G. Gray and V. H. Whittaker, *J. Anat.*, **96**, 79(1962).

(17) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265(1951).

(18) R. Baldessarini and M. Vogt, *J. Neurochem.*, **18**, 951(1971).

(19) G. A. Bray, *Anal. Biochem.*, **1**, 279(1960).

(20) K. J. Laidler, "The Chemical Kinetics of Enzyme Action," Clarendon Press, Oxford, England, 1958, p. 83.

(21) A. S. Burgen and L. L. Iversen, *Brit. J. Pharmacol. Chemother.*, **25**, 34(1965).

(22) J. E. Harris and R. J. Baldessarini, *Neuropharmacology*, **12**, 669(1973).

(23) R. M. Ferris, F. L. M. Tang, and R. A. Maxwell, *J. Pharmacol. Exp. Ther.*, **181**, 407(1972).

(24) J. H. Biel, in "Molecular Modification in Drug Design," Advances in Chemistry Series 45, American Chemical Society, Washington, D.C., 1964, p. 133.

(25) T. B. Vree, A. Muskens, and J. M. Van Rossum, *J. Pharm. Pharmacol.*, **21**, 774(1969).

(26) Y. Israel, F. J. Carmichael, and J. A. MacDonald, *Ann. N.Y. Acad. Sci.*, **215**, 38(1973).

(27) F. J. Carmichael and Y. Israel, *J. Pharmacol. Exp. Ther.*, **186**, 253(1973).

ACKNOWLEDGMENTS AND ADDRESSES

Received November 8, 1974, from the *Department of Pharmacology, Center for the Health Sciences, University of California at Los Angeles, Los Angeles, CA 90024*

Accepted for publication January 31, 1975.

Supported by U.S. Public Health Service Grants MHTP-6415 and MH-23839.

[x] To whom inquiries should be directed.

Flumizole, a New Nonsteroidal Anti-Inflammatory Agent

E. H. WISEMAN [x], H. M. McILHENNY, and J. W. BETTIS *

Abstract □ Flumizole is a potent anti-inflammatory agent in animal models with an inhibitory activity severalfold that of indomethacin in rat foot edema and prostaglandin synthetase tests. The drug was well absorbed from the GI tract when administered in the solution used in pharmacological assays. However, markedly poorer absorption of the solid form of this poorly water-soluble agent led to the development of a flumizole dispersion with polyethylene glycol 6000. The solid dispersion exhibited an increased dissolution rate in simulated gastric fluid and improved absorption properties in dogs relative to unformulated flumizole. Studies with a capsule formulation of the solid dispersion in human volunteers were indicative of good drug absorption. Plasma levels of flumizole were rapidly achieved and declined with a short half-life (2–7 hr) in rats, dogs, and humans.

Keyphrases □ Flumizole—anti-inflammatory activity, bioavailability, and formulation □ Anti-inflammatory agents—flumizole (nonsteroid) activity, bioavailability, and formulation □ Bioavailability—flumizole, a nonsteroidal anti-inflammatory agent □ Dissolution rates—formulations of flumizole

Flumizole[1] is one of a series of substituted diarylimidazoles with anti-inflammatory activity (1, 2). This compound is distinguished from the general class of acidic agents currently used in the management of inflammatory disease by its very weak acidic character (pKa 10.7). This report describes the potent anti-inflammatory activity of flumizole and biopharmaceutical developments undertaken to opti-

[1] 4,5-Bis(*p*-methoxyphenyl)-2-(trifluoromethyl)imidazole, synthesized by Dr. Joseph G. Lombardino, Department of Medicinal Chemistry, Pfizer Central Research, Pfizer, Inc., Groton, CT 06340