observations by us and others have shown that the addition of 5'-methylthioadenosine, the co-product of spermidine (and spermine) synthesis, to MEL cells inhibits differentiation without cytostasis [26, 27]. Further studies into the biochemical mechanisms involved in cell growth and gene expression are now necessary to better define these interrelationships.

In summary, the polyamines putrescine, spermidine and spermine are required for optimal cell growth and are implicated in differentiation. We have monitored the effects of AdoDATO, a transition-state analog and potent inhibitor of spermidine synthase, on the proliferation and differentiation of MEL cells. AdoDATO inhibited MEL cell proliferation in a concentration-dependent manner. Treatment of MEL cells with 125 μM AdoDATO for 24 hr resulted in a 2.9-fold increase in putrescine, a 95% decrease in spermidine and a 1.6-fold increase in spermine. The induction of MEL differentiation by dimethyl sulfoxide was inhibited by pretreatment with AdoDATO as monitored by benzidine staining, heme synthesis and globin RNA production. The addition of exogenous spermidine but not putrescine abrogated these inhibitory effects. These findings suggest that spermidine is necessary for MEL cell proliferation and differentiation.

*Laboratory of Clinical Pharmacology
Dana-Farber Cancer Institute
and
Harvard Medical School
Boston, MA 02115; and
†Department of Chemistry
Rensselaer Polytechnic Institute
Troy, NY 12180, U.S.A.

MATTHEW L. SHERMAN*
TIMOTHY D. SHAFMAN*
JAMES K. COWARD†
DONALD W. KUFE*‡

REFERENCES

1. C. W. Tabor and H. Tabor, *A. Rev. Biochem.* **53**, 749 (1984).
2. O. Heby, *Differentiation* **19**, 1 (1981).
3. B. W. Metcalf, P. Bey, C. Danzin, M. J. Jung, P. Casara and J. Vevert, *J. Am. chem. Soc.* **100**, 2551 (1978).
4. M. Sugiura, T. Shafman and D. Kufe, *Cancer Res.* **44**, 1440 (1984).
5. T. Watanabe, T. Shafman and D. Kufe, *J. cell. Physiol.* **122**, 435 (1985).
6. K-C. Tang, A. E. Pegg and J. K. Coward, *Biochem. biophys. Res. Commun.* **96**, 1371 (1980).
7. K-C. Tang, R. Mariuzza and J. K. Coward, *J. med. Chem.* **24**, 1277 (1981).
8. J. K. Coward, P. R. Chaudhari, M. A. Kwiat and M. Hluboky, *Fedn. Proc.* **42**, 2011 (1983).
9. A. E. Pegg, K-C. Tang and J. K. Coward, *Biochemistry* **21**, 5082 (1982).
10. S. H. Orkin, F. I. Harosi and P. Leder, *Proc. natn. Acad. Sci. U.S.A.* **72**, 98 (1985).
11. S. Sassa, *J. exp. Med.* **143**, 305 (1976).
12. T. Maniatis, E. F. Fritsch and J. Sambrook (Eds.), *Molecular Cloning* pp. 191–3. Cold Spring Harbor Laboratory, New York (1982).
13. P. S. Thomas, *Proc. natn. Acad. Sci. U.S.A.* **77**, 5201 (1980).
14. S. M. Tilghman, D. C. Tiemeier, R. Polsky, M. H. Edgell, J. G. Seidman, A. Leder, L. W. Enquist, B. Norman and P. Leder, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4406 (1977).
15. T. Oka, J. W. Perry, T. Takemoto, T. Sakaii, N. Terada and H. Inone, *Advances in Polyamine Research* (Eds. C. M. Calarera, V. Zappia and U. Bachrach), Vol. 3, pp. 309–320. Raven Press, New York (1981).
16. D. R. Bethell and A. E. Pegg, *Biochem. biophys. Res. Commun.* **102**, 272 (1981).
17. P. R. McClintock and J. Papaconstantinou, *Proc. natn. Acad. Sci. U.S.A.* **74**, 4551 (1971).
18. J. Levy, N. Terada, R. A. Rifkind and P. A. Marks, *Proc. natn. Acad. Sci. U.S.A.* **72**, 28 (1975).
19. A. W. Wiens, P. R. McClintock and J. Papaconstantinou, *Biochem. biophys. Res. Commun.* **70**, 824 (1976).
20. A. Leder, S. Orkin and P. Leder, *Science* **190**, 893 (1975).
21. R. Levenson, K. Kernen, A. Mitrani and D. Housman, *Devl. Biol.* **74**, 224 (1980).
22. Y. Tabuse, M. Kawamura and M. Furusawa, *Differentiation* **7**, 1 (1976).
23. J. Schindler, M. Kelly and P. P. McCann, *Biochem. biophys. Res. Commun.* **114**, 410 (1983).
24. J. Schindler, M. Kelly and P. P. McCann, *J. cell. Physiol.* **122**, 1 (1985).
25. M. Kelly, P. P. McCann and J. Schindler, *Devl Biol.* **111**, 510 (1985).
26. T. D. Shafman, M. L. Sherman and D. W. Kufe, *Biochem. biophys. Res. Commun.* **124**, 172 (1984).
27. P. DiFiore, M. Grieco, A. Pinto, V. Attadia, M. Porcelli, G. Cacciapuoti and M. Carteni-Farina, *Cancer Res.* **44**, 4096 (1984).

‡ Address correspondence and reprint requests to: Dr. Donald. W. Kufe, Dana-Farber Cancer Institute, 44 Binney St., Boston, MA 02115.

Biochemical Pharmacology, Vol. 35, No. 15, pp. 2636–2639, 1986.
Printed in Great Britain.

0006-2952/86 $3.00 + 0.00
Pergamon Journals Ltd.

Concentrations of the enantiomers of 5-hydroxymethtryptoline in mammalian urine: implications for *in vivo* biosynthesis

(*Received* 26 *August* 1985; *accepted* 31 *January* 1986)

5-Hydroxymethtryptoline (5-HMTLN, Fig. 1) represents the Pictet–Spengler condensation [1] product of serotonin and acetaldehyde and belongs to a class of compounds (β-carbolines) which have long been known to occur naturally in plants [2]. Members of the class have physiological and pharmacological effects in mammalian systems, including inhibition of the monoamine oxidase enzymes [3, 4], inhibition of monoamine reuptake mechanisms [5] and affinity for various monoamine binding sites [6–8]. Because acetaldehyde is a metabolite of ethanol, the presence of 5-HMTLN in mammals was originally implicated in connection with ethanol intake.

Surprisingly, however, 5-HMTLN was found to be a normal constituent of human urine [9], and rat tissues and body fluids [10]. 5-HMTLN exists as two stereoisomers because of the chiral carbon atom in the position which is created in the formation of the tricyclic structure (Fig. 1). Formation from a stereoselective process would be reflected in an unequal abundance of the two enantiomers and could be indicative of the direct participation of an

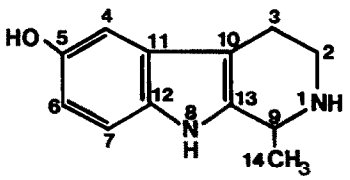

Fig. 1. Chemical structure of 5-hydroxymethtryptoline (5-HMTLN, also known as 6-hydroxy-1-methyltetrahydro-β-carboline) showing the numbering system. The chiral carbon atom is located at position 9.

enzymatically mediated biosynthetic reaction. We sought to take advantage of the chiral nature of 5-HMTLN by examining urine extracts from a number of mammalian species for the relative concentrations of the two isomers. To achieve this, we used a GC/MS method employing a chiral capillary column which allowed independent measurement of the enantiomers of 5-HMTLN.

Experimental

Chemicals. 5-HMTLN HCl and the internal standard 5-hydroxymeth(2,2,3,3-$^2H_4$)-tryptoline HCl (5-HMTLN-$^2H_4$) was prepared from 5-benzyloxytryptamine HCl and 5-benzyloxy-($\alpha,\alpha,\beta,\beta$-$^2H_4$) tryptamine HCl, respectively [11]. 5-Hydroxy-($\alpha,\alpha,\beta,\beta$-$^2H_4$) tryptamine picrate and 5-benzyloxy-($\alpha,\alpha,\beta,\beta$-$^2H_4$) tryptamine HCl was prepared according to Shaw *et al.* [12]. 5-Hydroxytryptamine hydrogen oxalate was obtained from Calbiochem (Los Angeles, CA); sulphatase (type H1) containing β-glucuronidase activity from Sigmal Chemical Co. (St. Louis, MO); pentafluoropropionic anhydride (PFPA) from Regis Chemical Co. (Morton Grove, IL); and dichloromethane from Mallinkrodt Inc. (Paris, KY). Other chemicals used were of analytical purity.

Urine samples. Single urine samples were collected in polypropylene tubes from rat (Sprague–Dawley, 250 g), mouse (Balb/C, 20 g), cat, dog, squirrel monkey and man either by direct pressure of the bladder or by collecting the urine from a single urination. The urine was immediately frozen and stored at −70°. Twenty-four hour urine samples from rat and mouse were collected using metabolic cages. In this case the plastic vessel collecting the urine was kept over dry ice resulting in almost immediate freezing of the fluid following urination.

In one experiment, 24 hr urine samples were collected from 4 male rats under normal conditions (control) and during starvation. In this experiment the animals subjected to the starvation protocol were food-deprived for 12 hr before the commencement of urine collection. Urine from axenic (germ-free) rats was collected at the Lobund Laboratories, University of Notre Dame, IN.

GC–MS analysis. Aliquots of urine (1.0 ml) were pipetted into 15 ml acid-washed (dichromate–sulphuric acid) silanized glass tubes containing in a total volume of 500 μl: 5-HMTLN-$^2H_4$ (internal standard, 108 pmole); semicarbazide (3.8 μmole); citrate buffer (0.25 μmoles in 0.5 ml H_2O, pH 6.0) and sulphatase (2.5 mg). Following incubation at 37° for 16 hr, the samples were extracted with CH_2Cl_2 (5 ml) and the organic layer discarded. Thereafter solid NaCl (0.4 g), aqueous potassium carbonate (100 μl, 5.8 M) and 5 ml of additional CH_2Cl_2 were added, and the tubes were shaken and centrifuged at 1000 *g* for 5 min. The organic layers were transferred to clean silanized glass test-tubes and evaporated to dryness under a stream of nitrogen. The residues were treated with 100 μl of PFPA for 30 min at 60°. After cooling, the excess reagent was evaporated under a stream of nitrogen and the residues were redissolved in 100 μl of EtOAc prior to analysis by GC/MS.

A modified Hewlett-Packard 5985B gas chromatograph–mass spectrometer was used for the recording of mass spectra and multiple ion detection. Gas chromatographic separations were achieved with a solvent-free injector (dropping-needle type, Ray Allen Assoc., Boulder, CO) connected to a capillary column using helium as a carrier gas. Separation of the enantiomers of the PFP derivatives of 5-HMTLN and 5-HMTLN-$^2H_4$ was achieved with a Chirasil-Val III Heliflex fused silica capillary column (25 m, 0.31 mm i.d., film thickness 0.23 μm, Applied Sciences, State College, PA). The column ends were inserted directly into the ion source of the mass spectrometer and a pressure of 0.4 atm over atmosphere of helium was maintained in the injector port, resulting in flowrates of 1–2 ml/min at 20°. The injector port and transfer line was maintained at 250 and 240°, respectively. The GC oven was kept at a temperature of 180° for 1 min following injection, then increased at a rate of 10° per min to a final temperature of 220°.

The mass spectrometer was operated in the negative ion chemical ionization mode (NCI) with methane as the reagent gas. The reagent gas was delivered to the source of the mass spectrometer coaxially with the capillary column and a pressure of approximately 1 Torr was maintained in the ionization chamber. The ion source and analyzer sections of the instrument were maintained at 100° and 210°, respectively, and an emission current of 300 μA was used with an electron energy of 230 eV.

Quantitative data were obtained in the multiple ion detection mode by recording the relative intensities of the ions at *m/z* 474 (5-HMTLN-diPFP) and 478 (5-HMTLN-$^2H_4$-diPFP). Calculation of the quantity of 5-HMTLN in each sample was made by interpolation from calibration curves constructed from standard samples. These curves were constructed by plotting the ion current ratios (*m/z* 474/478) against the concentration of the unlabelled isomer.

Results and discussion

The predominant products (>90%) formed by treatment of synthetic 5-HMTLN and 5-HMTLN-$^2H_4$ with PFPA were identified as the diPFP species. Under electron capture CI conditions these derivatives showed base peaks at *m/z* 346 and 350 representing the loss of a CF_3CF_2COH group from the corresponding molecular ions. Less intense, but abundant ions (30%), formed by loss of HF from the molecular ions, were observed at *m/z* 474 and 478. Because of the greater diagnostic value of these higher mass fragments, they were chosen for monitoring in the selected ion monitoring mode.

Under the conditions employed, the Chirasil-Val III fused silica capillary column produced a base-line separation of the two enantiomers of 5-HMTLN-diPFP with retention times of between 8.5 and 9 min. The two peaks were designated A and B according to the order in which they eluted. Synthetic 5-HMTLN and 5-HMTLN-$^2H_4$ gave chromatograms in which peaks A and B were always of equal height and area. When urine extracts were analyzed, however, the two peaks were typically of unequal abundance. With the chiral capillary column, the two peaks obtained from urine extracts in the *m/z* 474 ion trace had identical retention times as the two peaks obtained from authentic 5-HMTLN-diPFP. Representative ion trace chromatograms obtained from the analysis of cat and rat urine extracts are shown in Fig. 2. In both these species, the A-enantiomer predominates.

To exclude the possibility that the analytical method discriminated between the enantiomers, an experiment was performed in which three human urine samples were spiked with racemic 5-HMTLN (110.2 pmol) prior to workup. The results showed an increase in the spiked samples of 58.1 pmol of each of the A and B isomers, confirming the validity of the analytical procedure for providing independent and reliable estimates of the urinary con-

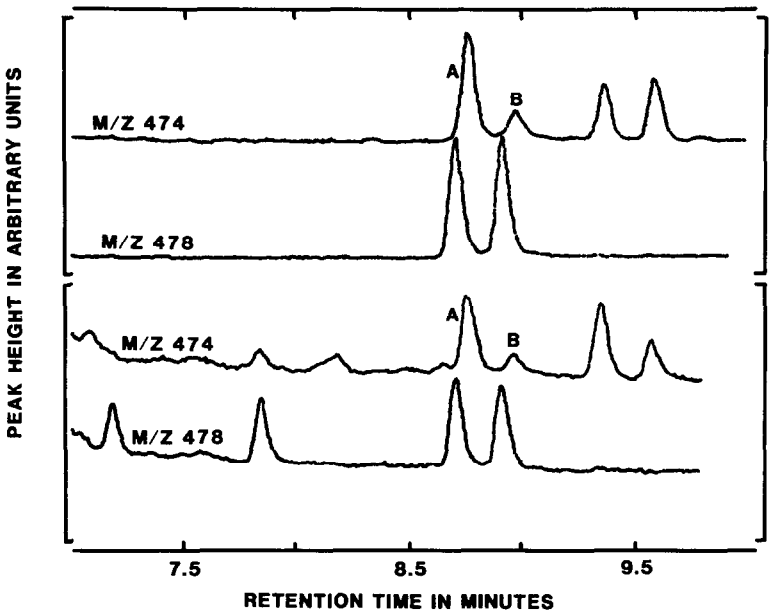

Fig. 2. Ion traces obtained from the mass spectrometric analysis of rat (upper) and cat (lower) urine extract. The peaks representing the enantiomers of 5-HMTLN are marked A and B on the traces for *m/z* 474. The corresponding peaks for the internal standard are seen on the traces for *m/z* 478 showing the slightly shorter retention times due to the presence of four deuterium atoms.

centrations of each enantiomer. We were also conscious of the possibility that the different relative abundances observed for the isomers of 5-HMTLN could be a consequence of stereoselective metabolism or excretion of 5-HMTLN. To further test the accuracy of the analytical method, racemic 5-HMTLN was administered (i.p.) to rats at two different doses, and 24 hr urine specimens were collected (Table 1). The enantiomers were recovered in urine in equal abundance, both at the lower dose which increased the urinary output of 5-HMTLN about 100-fold, and at a higher dose which increased the output about 2000-fold. This result showed that at least in rat, where the A and B isomers normally occur in unequal abundance, a different rate of metabolism or excretion of the two isomers is unlikely.

Two approaches were used to check the possibility of artifactual formation of 5-HMTLN during sample work-up by spontaneous condensation of 5-hydroxytryptamine with acetaldehyde present in the urine or introduced as a trace contaminant in solvents. In the first approach we documented the quantity of 5-HMTLN in the final derivatized sample resulting from the extraction of an aqueous solution of serotonin (3 nmol in 1 ml). This test was applied when any change in the work-up procedure was undertaken (e.g. when a new bottle of solvent was used). In the second approach we documented the quantity of 5-HMTLN-$^2H_4$ in the final derivatized sample resulting from the extraction of a duplicate urine sample in which serotonin-$^2H_4$ (1 nmol) was substituted for the internal standard. In neither of these tests was any evidence ever obtained for any formation of 5-HMTLN from serotonin during the work-up procedure.

A dietary origin of 5-HMTLN is unlikely since we were unable to detect it in rat chow (<1 pmol/g). In addition, rats starved for 36 hr continued to excrete the metabolite in significant quantities, 9.05 ± 2.25 (S.E.M., N = 4) pmol/ml of the A-isomer and 3.33 ± 0.64 (S.E.M., N = 4) pmol/ml of the B-isomer. In the absence of an additional source of the compound, the relatively short *in vivo* half-life of 1–2 hr [10] would have been more than sufficient for complete clearance from the body within the time frame of the food deprivation experiment. The concentration of 5-HMTLN in the urine from the starved rats was between 14 and 24% of the concentration found in the urine from fed animals. This result is not unexpected because of the decreased metabolism during starvation. Earlier work has shown that tryptophan is the ultimate precursor of this compound and decreased tryptophan intake results in a significant decrease in urinary output of the metabolite [13]. The presence of 5-HMTLN in urine from germ-free rats, at a concentration of 11.1 ± 4.4 (S.E.M., N = 4) pmol/ml of the A-isomer and 1.51 ± 0.12 (S.E.M., N = 4) pmol/ml of the B-isomer, indicates that the compound is not a metabolite produced

Table 1. Relative abundance of the enantiomers of 5-HMTLN recovered in rat urine following i.p. administration

Treatment	Animal No.	Ratio A isomer/B isomer	A plus B (nmol/24 hr)
Control	1	2.06	0.23
	2	2.11	0.20
41.9 nmol i.p.	3	0.97	22
	4	1.07	22
839 nmol i.p.	5	0.96	504
	6	1.04	234

by the intestinal flora. This is in agreement with the low concentrations of 5-HMTLN previously found in the intestine [10].

Measurements of the concentrations of the A and B forms of 5-HMTLN in enzymatically hydrolyzed urine from six mammalian species revealed an unequal abundance of the enantiomers in urine from rat, cat and squirrel monkey, whereas in human, dog and mouse the enantiomers occurred in approximately equal abundance (Table 2). The range of concentrations of 5-HMTLN observed for the different individuals in each species was relatively narrow for rat, cat, mouse and monkey, but varied widely for dog and humans. However, in these species there seemed to be no correlation between the relative abundance of the two enantiomers and their combined concentrations.

Table 2. Relative abundance of the enantiomers of 5-HMTLN occurring in urine of mammalian species

Species	Ratio A/B (mean ± S.E.M.)	Conc. range (pmol/ml)	N
Rat	3.40 ± 0.5	52.0 ± 86	7*
Cat	7.60 ± 0.5	30.0 ± 90	7
Squirrel monkey	0.59 ± 0.05	90.0 ± 494	6
Mouse	0.94 ± 0.07	30.0 ± 137	7*
Dog	0.92 ± 0.11	2.3 ± 121	8
Human	0.97 ± 0.05	84.6 ± 2380	9

* Some samples were pooled from several individuals.

This study confirms and considerably extends earlier reports describing the existence of 5-HMTLN in mammalian urine [9, 10]. The concentrations of 5-HMTLN found in rat and human urine are in good agreement with the previous reports, and for the first time 5-HMTLN was identified and quantified in cat, dog, mouse and squirrel monkey urine. Furthermore, through the use of a chiral capillary gas chromatographic column, we were able to independently quantify the two enantiomers of 5-HMTLN. Both enantiomers of 5-HMTLN were found in the urine of all species investigated, although in three of these species the enantiomers occurred in unequal abundance. Surprisingly, in rat and cat urine a different enantiomer predominated than in squirrel monkey urine. The different amounts of the two enantiomers in rat, cat and squirrel monkey urine suggest that in these species at least, an enzymatic process must be involved.

While details of the biosynthetic route of urinary 5-HMTLN are at this time unclear, it would seem appropriate to briefly speculate about possible intermediates in the biosynthetic pathway. Formation from serotonin and acetaldehyde would seem unlikely under normal conditions because of the low *in vivo* concentrations of acetaldehyde [14, 15]. Alternatively, pyruvate could function as the carbonyl reactant [16], and subsequent decarboxylation would yield 5-HMTLN. Another possibility is that 5-HMTLN arises from hydroxylation of the analogue methtryptoline (1-methyl-tetrahydro-β-carboline). This route would be analogous to the recently reported *in vivo* hydroxylation of tryptoline (tetrahydro-β-carboline) at both the 5 and 6 positions in rats [17].

In summary, the enantiomers of 5-HMTLN were independently assayed in urine by GC-MS using a chiral capillary column and electron capture chemical ionization. The enantiomers were found in about equal abundance in urine from man, dog and mouse, whereas in rat, cat and squirrel monkey there was a predominance of one over the other. The results suggest the *in vivo* formation of 5-HMTLN proceeds via an enzymatically assisted reaction.

Acknowledgements—The authors gratefully acknowledge the support of Dr. J. D. Barchas for his encouragement in pursuing this work and the cooperation of Dr. Morris Pollard, Director of the Lobund Laboratories, University of Notre Dame, for the collection of urine from axenic rats. Supported by a grant to J. D. Barchas from the National Institute on Alcohol Abuse and Alcoholism (AA 05972).

Pasarow Analytical Neurochemical Facility
Nancy Pritzker Laboratory of Behavioral Neurochemistry
Department of Psychiatry and Behavioral Sciences
Stanford University School of Medicine
Stanford, CA 94305, U.S.A.

OLOF BECK*
KYM F. FAULL

* Author to whom correspondence should be sent. Present address: Department of Toxicology, Karolinska Institutet, Box 60400, S-104 01 Stockholm, Sweden.

REFERENCES

1. W. M. Whaley and T. R. Govindachari, in *Organic Reactions*, Vol 6 (Eds. R. Adams, H. Adkins and A. M. Cope), p. 151. Wiley, New York (1951).
2. J. R. F. Allen and B. Holmstedt, *Phytochemistry* **19**, 1573 (1980).
3. N. S. Buckholtz and W. O. Boggan, *Biochem. Pharmac.* **26**, 1991 (1977).
4. M. B. H. Youdim and B. Oppenheim, *Neuroscience* **6**, 801 (1981).
5. K. J. Kellar, G. R. Elliott, R. B. Holman, J. Vernikos-Danellis and J. D. Barchas, *J. Pharmac. exp. Ther.* **198**, 619 (1976).
6. M. M. Airaksinen and I. Kari, *Med. Biol.* **59**, 190 (1981).
7. C. S. Cascio and K. J. Kellar, *Neuropharmacology* **21**, 1291 (1982).
8. M. Nielsen and C. Braestrup, *Nature, Lond.* **286**, 606 (1980).
9. O. Beck, T. R. Bosin, A. Lundman and S. Borg, *Biochem. Pharmac.* **31**, 2517 (1982).
10. O. Beck and A. Lundman, *Biochem. Pharmac.* **32**, 1507 (1983).
11. R. G. Taborsky and W. M. McIsaac, *J. Med. Chem.* **7**, 135 (1964).
12. G. J. Shaw, G. J. Wright and G. W. A. Milne, *Biomed. Mass Spectrom.* **3**, 146 (1976).
13. O. Beck, K. F. Faull, J. D. Barchas, J. V. Johnson and R. A. Yost, in *Acetaldehyde Adducts in Alcoholism* (Ed. M. A. Collins), p. 145. A. R. Liss, New York (1985).
14. C. J. P. Eriksson and R. A. Dietrich, *Pharmac. Biochem. Behav.* **13**, Suppl. 1, 291 (1980).
15. C. J. P. Eriksson, *Biochem. Pharmac.* **34**, 3979 (1985).
16. A. Brossi, in *Beta-Carbolines and Tetrahydroisoquinolines* (Eds. F. Boom, J. Barchas, M. Sandler and E. Usdin), p. 125. A. R. Liss, New York (1982).
17. B. Greiner and H. Rommelspacher, *Naunyn-Schmiedeberg's Archs Pharmac.* **325**, 349 (1984).