Biochemical Pharmacology, 1965, Vol. 14, pp. 699–702. Pergamon Press Ltdl, Printed in Great Britain.

IN VITRO PRECIPITATION OF LIVER MICROSOMES BY DEPTROPINE AND SOME STRUCTURALLY RELATED COMPOUNDS: A CAUSE FOR THE DISCREPANCIES OBSERVED BETWEEN *IN VIVO* AND *IN VITRO* *N*-DEMETHYLATION

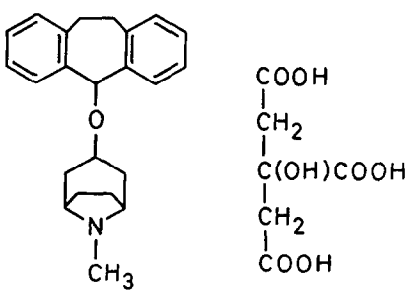
R. C. ROOZEMOND, G. J. B. VEGT,* W. HESPE and W. TH. NAUTA

Research Department of N. V. Koninklijke Pharmaceutische Fabrieken v/h Brocades-Stheeman en Pharmacia, Amsterdam, The Netherlands

(*Received* 5 *October* 1964; *in revised form* 21 *November* 1964; *accepted* 2 *December* 1964)

Abstract—Deptropine, 3α-(9,10-dihydro-5*H*-dibenzo[*a*, *d*]cyclohepten-5-yloxy) tropane, and some structurally related compounds were found to associate with and to precipitate rat liver microsomes *in vitro*. This provides an explanation for the following findings: *in vitro* deptropine is poorly demethylated; it inhibits the demethylation of other substrates; concentrations upwards of about $3 \cdot 10^{-4}$ M exert an inhibitory effect on the NADPH-oxidase in suspensions of rat liver microsomes. However, the drug is well demethylated in *intact* rats: within 6 hr after intraperitoneal administration more than 22 per cent is metabolised by this route.

IN PREVIOUS publications[1, 2] the synthesis and pharmacological properties have been reported of 3α-(9,10-dihydro-5*H*-dibenzo[*a*,*d*]cyclohepten-5-yloxy)tropane citrate. It is used under the WHO-proposed name of deptropine citrate (Brontine®) in the therapy of chronic non-specific respiratory diseases.



Deptropine Citrate

Chromatographic analysis of urine revealed that *in vivo* this drug is subject to hydrolysis and demethylation.[3] It has been shown by many investigators that oxidative *N*-demethylation constitutes one of the most important metabolic pathways of aliphatic and aromatic methyl-substituted amines in man and animal. In the case of substances

* Present address: Laboratory for Medical-Veterinary Chemistry of the Rijksuniversiteit at Utrecht.

that are alien to the body this demethylation is catalysed by an enzyme system localised in the microsomes of liver cells.[4–7] For this enzyme system to exert its activity it requires oxygen and a source of reduced nicotinamide adenine dinucleotide phosphate. The present publication describes a further investigation of *in vitro* and *in vivo* demethylation of deptropine and its interaction with rat liver microsomes.

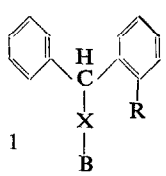
Although initial *in vitro* experiments led us to conclude that deptropine has an inhibitory effect on the demethylating enzyme system in rat liver microsomes, *in vivo* the compound was found to suffer a rather rapid demethylation. Continued investigation of the *in vitro* behaviour of deptropine with respect to liver microsomes and the NADPH-oxidase in these particles showed that the enzyme inhibition observed in the initial *in vitro* experiments must have been an artifact. Evidence was obtained that deptropine and some structurally related compounds are able to associate with and precipitate liver microsomes *in vitro*, thus eliminating the enzymes localised in the microsomes from the reaction medium. To facilitate some experiments deptropine (-tropanyl-3-^{3}H) was used in the *in vitro*, deptropine(-N-$^{14}CH_3$) in the *in vivo* investigations.

MATERIALS AND METHODS

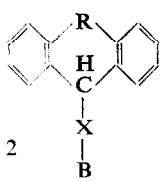
Chemicals

The compounds used in the present investigation are listed in Table 1. Most of these have been synthesised as novel substances at our laboratories.

TABLE 1. STRUCTURALLY RELATED COMPOUNDS, USED IN THE PRESENT INVESTIGATION



1



2

	Formula	R	X	B
Diphenhydramine	1	H	O	β-dimethylaminoethyl
Orphenadrine	1	CH_3	O	β-dimethylaminoethyl
BS 6534	1	tert-C_4H_9	O	β-dimethylaminoethyl
Benztropine	1	H	O	3α-tropanyl
2-Methylbenztropine	1	CH_3	O	3α-tropanyl
BS 6762	2	—CH_2—CH_2—	O	β-dimethylaminoethyl
BS 7051	2	—CH_2—CH_2—	O	1-methyl-4-piperidyl
BS 7369	2	—CH_2—CH_2—	O	1-methyl-3-pyrrolidyl
Deptropine	2	—CH_2—CH_2—	O	3α-tropanyl
BS 6763	2	—CH=CH—	O	β-dimethylaminoethyl
BS 7039	2	—CH=CH—	O	3α-tropanyl
BS 7301	2	—$(CH_2)_3$—	O	β-dimethylaminoethyl
BS 7006	2	—CH_2—CH_2—	COO	β-dimethylaminoethyl
BS 7042	2	—CH_2—CH_2—	COO	3α-tropanyl

Imipramine and atropine were included in one of the experiments on account of their structural resemblance to deptropine. The synthesis of the radioactive-labeled compounds deptropine(tropanyl-3-^{3}H)-specific activity: 18 mc/g-and deptropine(-N-$^{14}CH_3$)-specific activity: 0·31 mc/g-will be described elsewhere.

In vitro *demethylations*

The procedure described by McMahon[8] and McMahon and Easton[9] was adopted for determining the amount of formaldehyde formed during *in vitro* demethylation.

Incubations were carried out with the supernatant of rat liver homogenate in four parts of ice-cold 0·1 M phosphate buffer (pH 7·4), centrifuged at 13,000 *g*. To an aliquot of the supernatant, corresponding to 6 g of rat liver tissue were added: 225 μmoles of semicarbazide, 675 μmoles of nicotinamide, 450 μmoles of magnesium chloride, 300 μmoles of glucose-6-phosphate, and sufficient 0·1 M phosphate buffer (pH 7·4) to bring the final volume to 32 ml. The composition of our mixture differed from that used by McMahon and yielded better results.

Two portions of 15 ml were transferred to 100-ml flasks containing 6·5 μmoles NADP. After 5 min equilibration at 37° 1 ml of 0·1 M phosphate buffer (pH 7·4) was placed in the one flask (blank incubation mixture) and a solution of the compound under test in 1 ml of the same buffer in the other. At different intervals after substrate addition 3 ml aliquots were removed and assayed on formaldehyde.[8, 9]

In vivo *demethylation of deptropine in the rat*

Male albino rats (TNO-WU strain) of 150–200 g were used to study the *in vivo* demethylation of deptropine(-N-$^{14}CH_3$). After administration of the radioactive substance the rats were placed in a metabolism cage based on the design by Roth *et al.*[10] The expiratory air was passed through an absorption unit of the type described by Edwards *et al.*[11] The radioactive carbon dioxide was bound directly into an ethanolamine-containing scintillation cocktail:[12] amounts of 50 ml in each of the two connected absorption vials were found to suffice for quantitatively binding the carbon dioxide expired during one hr. After mixing of the absorption tube contents and pipetting 15 ml aliquots into standard polyethylene counting vials, the radioactivity was measured with the aid of a Packard Tricarb. Scintillation Spectrometer, type 314EX. If necessary, corrections for quenching were made by a channel ratio method.[13, 14]

The radioactive deptropine citrate was given by the intraperitoneal route at a dose of 10 mg/kg of body weight. In some instances the rats had been pretreated with SKF 525-A, a well-known inhibitor of the demethylating enzyme system in liver microsomes (25 mg/kg, administered intraperitoneally 45 min before deptropine citrate).

Preparation of rat liver microsome suspensions

To investigate the effect of drugs on liver microsomes and to determine the NADPH-oxidase activity we used suspensions of rat liver microsomes in phosphate buffer, prepared according to Gillette *et al.*[15] Starting from 10 g of rat liver, suspensions were prepared in a final volume of 100 ml of 0·1 M phosphate buffer, pH 7·4.

RESULTS AND DISCUSSION

In vitro *demethylations*

As substrates for *in vitro* demethylation studies were used: diphenhydramine, orphenadrine, BS 6534, benztropine, deptropine and *N*-methylaniline. The results of the experiments are illustrated in Fig. 1.

It is remarkable that after a rapid onset the demethylation of deptropine at a concentration of 10^{-3} M soon decreases and after five minutes is considerably less

than that of deptropine at a concentration of 10^{-4} M. The structurally related benztropine, too, shows a slow demethylation when compared with orphenadrine, BS 6534 and diphenhydramine.

To check whether deptropine inhibits the demethylation of other substrates, the compound was added to the incubation mixture one minute before diphenhydramine,

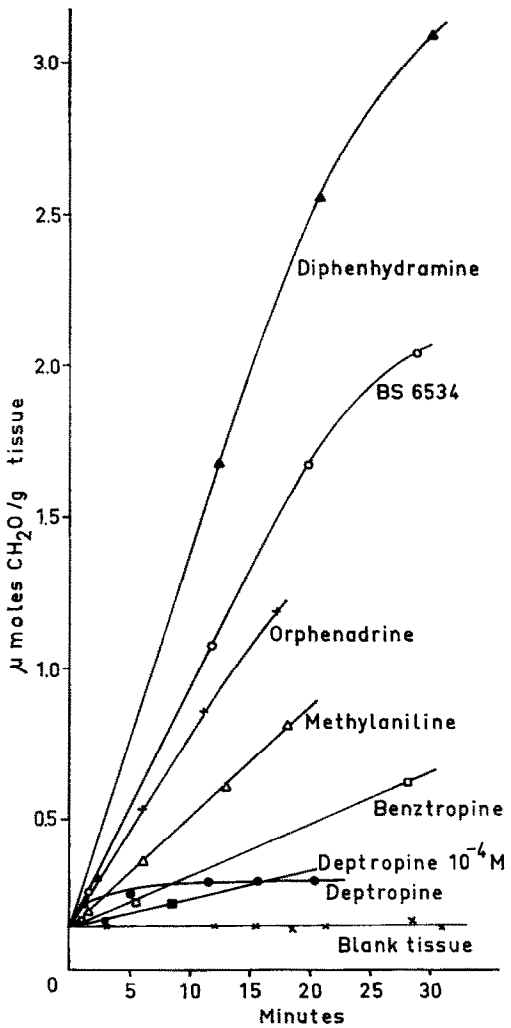


FIG. 1. *In vitro* demethylation of deptropine and some other compounds by rat liver microsomes (concentration of compounds 10^{-3} M unless stated otherwise).

which is a good substrate for the demethylating enzyme system. The test concentration of diphenhydramine was 10^{-3} M. It was found, that deptropine at concentrations of 10^{-4} M and $8 \cdot 10^{-4}$ M inhibits the demethylation of diphenhydramine by 21 per cent and 56 per cent, respectively, determined after 15 min.

It is clear from the above experiments that deptropine (at least from a concentration of 10^{-4} M upwards) has an inhibitory effect on the demethylating enzyme system of rat liver microsomes *in vitro*.

Determination of in vitro *NADPH-oxidase activity in liver microsomes*

Microsomal *N*-dealkylation involves NADPH-oxidation, which is catalysed by the enzyme NADPH-oxidase.[7, 15] This oxidation can be followed spectrophotometrically at 340 mμ.[15]

Attempts were made to find out whether the deptropine-induced inhibition of the demethylating enzyme system in liver microsomes *in vitro* was attributable to an inhibition of the NADPH-oxidase. In these experiments use was made of microsomal suspensions obtained from rat liver. After addition of different amounts of deptropine to incubation mixtures prepared from the microsomal suspensions we noticed from a definite concentration onwards a turbidity increasing with time until a constant value had been reached. Especially during the first minute the turbidity increased rapidly: at a concentration of $6 \cdot 10^{-4}$ M the optical density, measured at 340 mμ, rose from 0 to 1·4 (see Fig. 2).

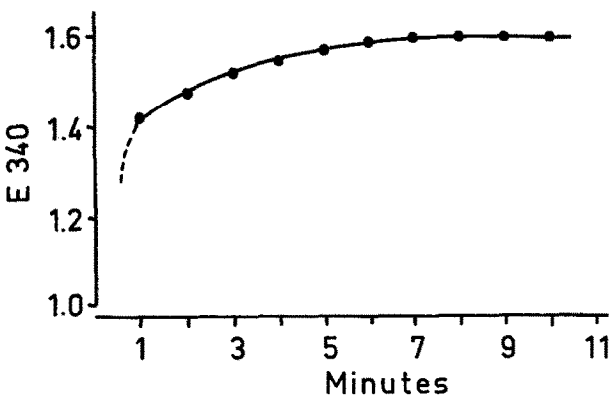


FIG. 2. Optical density at 340 mμ of a rat liver microsomal suspension after addition of $6 \cdot 10^{-4}$ M deptropine.

This intriguing phenomenon induced us to determine the rise in optical density at 340 mμ after addition of deptropine, in different concentrations, to microsomal suspensions. To check whether structurally related compounds show the same behaviour, determinations were carried out with diphenhydramine, BS 7051 and BS 7042. The last two compounds resemble deptropine in its dibenzocycloheptene structure and qualitatively they have the same effect on microsome suspensions as deptropine (Fig. 3). The turbidity decreases in the order: deptropine, BS 7042, BS 7051. The effect of diphenhydramine is negligible.

The NADPH-oxidase activity was now determined in suspensions of rat liver microsomes, using different deptropine concentrations, after separation of the precipitates obtained under influence of this drug. It is clear from Fig. 4, that the NADPH-oxidase is initially stimulated at a deptropine concentration of $2 \cdot 10^{-4}$ M, but the enzyme activity rapidly diminishes with increasing deptropine concentrations; at a concentration of $7 \cdot 10^{-4}$ M the enzyme activity is only 12% of that of the blank.

In interpreting these results it should be taken into account that the decreasing NADPH-oxidase activity at increasing deptropine concentrations can be attributed

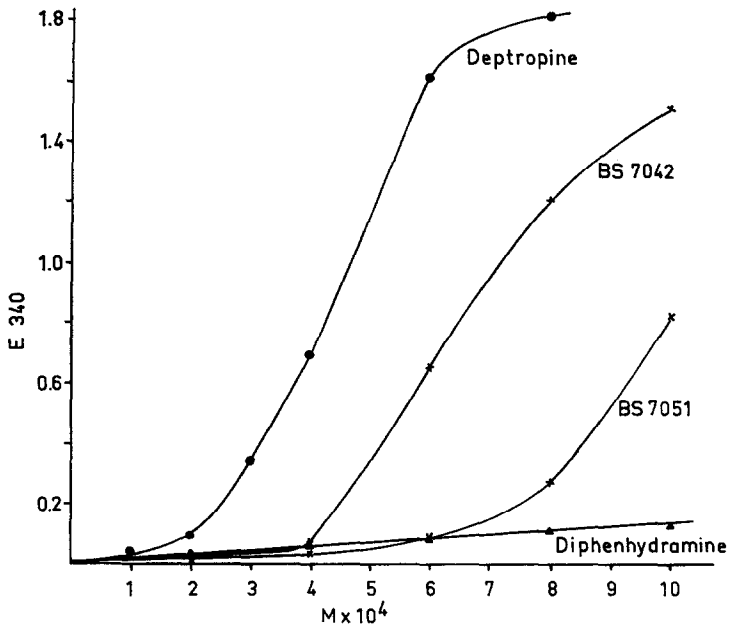


FIG. 3. Optical density at 340 mμ of a rat liver microsomal suspension 20 min after addition of different amounts of deptropine and structurally related compounds.

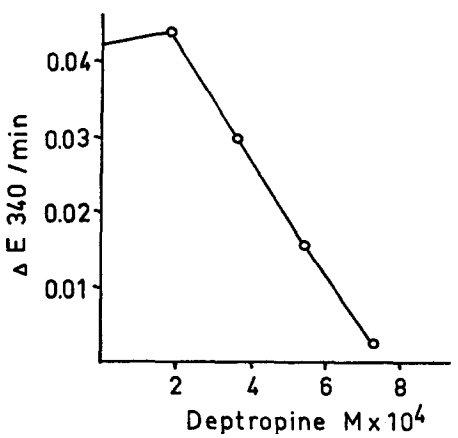


FIG. 4. NADPH-oxidase activity in rat liver microsomal suspensions containing different amounts of deptropine.

Conditions: Amounts of 5 ml were taken from rat liver microsomal suspensions (see Materials and Methods) containing 675 μmoles of nicotinamide and 450 μmoles of $MgCl_2$ per 60 ml. After addition of deptropine citrate dissolved in 1 ml of 0·1 M phosphate buffer (pH 7·4) and leaving the suspensions overnight at 0° they were centrifuged at 2000 *g*. In the supernatant NADPH-oxidase activity was determined spectrophotometrically after addition of a little NADPH to the cuvette containing the supernatant: the change in optical density was taken as a criterium for the rate of oxidation.

to the elimination of microsomes from the incubation medium due to precipitation by deptropine (see Fig. 3).

The microsome-precipitating effect of deptropine

We have attempted to answer the question whether microsome precipitation in *in vitro* experiments is responsible for the reduced NADPH-oxidase activity and diminished demethylation by determining the amount of microsomes in the supernatant after addition of deptropine (see Fig. 5). The micro Kjeldahl procedure proved more accurate than the micro method for protein determination.

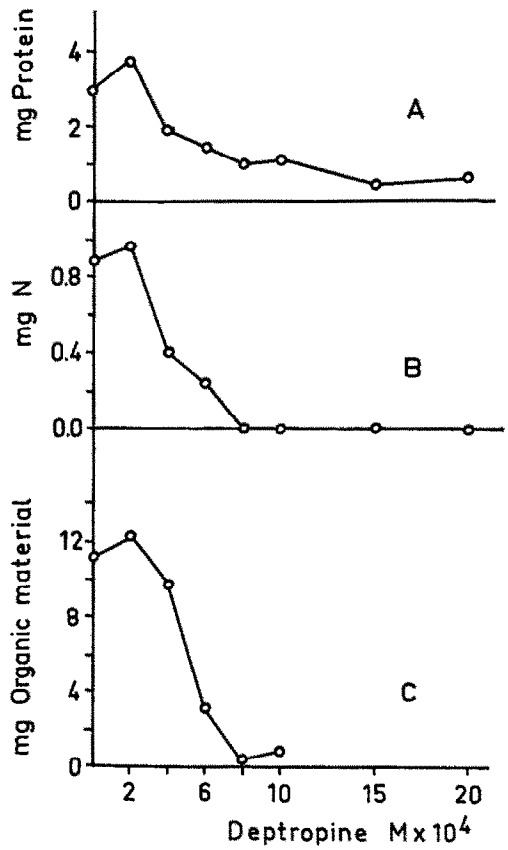


FIG. 5. A. Protein content of supernatants of rat liver microsomal suspensions after addition of different amounts of deptropine and subsequent centrifuging at 2000 *g*.

B. Nitrogen content in microsome sediment after addition of different amounts of deptropine to rat liver microsomal suspensions and removing the initially formed precipitate (see A).

C. Content of organic material in microsome sediment (see B).

Conditions (A, B, C): Different amounts of deptropine citrate dissolved in 1 ml of 0·1 M phosphate buffer (pH 7·4) were added to 9 ml aliquots of a rat liver microsomal suspension. After leaving overnight at 0° the suspensions were centrifuged at 2000 *g*. The protein content was determined in 0·4 ml of the supernatant by a micro procedure described by Mattenheimer.[19] The rest of the supernatant was centrifuged at 90,000 *g* for one hour. In the sediments the nitrogen content was determined by a micro Kjeldahl procedure and the amount of organic material according to Johnson.[20]

Figure 5 shows an *apparent* initial increase in the amount of organic material. This fallacy can be explained by assuming that, initially, deptropine associates with microsomes, but does not yet precipitate them. The colouring, more intense than was expected, in the protein determination, at a deptropine concentration of $2 \cdot 10^{-4}$ M, suggests that deptropine has a demasking effect on peptide bonds; this would agree with the increased NADPH-oxidase activity in the presence of $2 \cdot 10^{-4}$ M deptropine (see Fig. 4).

In order to investigate the possibility of coprecipitation of deptropine in suspensions of rat liver microsomes we used labeled deptropine, i.e. deptropine(-tropanyl-3-^{3}H). Table 2 shows that deptropine is indeed coprecipitated with the microsomes.

TABLE 2. COPRECIPITATION OF DEPTROPINE WITH MICROSOMES FROM RAT LIVER MICROSOMAL SUSPENSIONS AT DIFFERENT CONCENTRATIONS OF DEPTROPINE*

Deptropine M	Deptropine in sediment μg	Deptropine in supernatant† μg	Ratio deptropine in sed. to deptropine in supernatant %
$2 \cdot 10^{-4}$	0·6	400	0·15
$4 \cdot 10^{-4}$	9·4	790	1·19
$6 \cdot 10^{-4}$	20·4	1180	1·73
$8 \cdot 10^{-4}$	47·9	1550	3·09
10^{-3}	104·8	1900	5·52

* Conditions: Amounts of 1 ml of deptropine(-tropanyl-3-^{3}H) solutions were added to 5 ml aliquots of a rat liver microsomal suspension (see Materials and Methods). After leaving overnight at 0° the suspensions were centrifuged at 2000 *g* and the sediments washed twice with 5 ml of 0·1 M phosphate buffer (pH 7·4). After refluxing the sediments with 2 ml of 1 N NaOH and addition of hyamine solution to make up to a total volume of 10 ml the radioactivity was measured with the aid of a Packard Tri-Carb. Scintillation Spectrometer, type 314EX. By repeating the measurements after addition of an internal standard the quantities of deptropine could be calculated.

† Obtained after subtraction of the amount of deptropine found in the sediment.

For the area between deptropine concentrations $4 \cdot 10^{-4}$ and 10^{-3} M a linear relationship could be deduced between the logarithm of the amount of coprecipitated deptropine and the initial deptropine concentration. No linear relationship was found to exist between the logarithms of the two variables; this suggests that there is no pure adsorption (see Freundlich)[16]. Hence, we may conclude that deptropine associates with microsomes, though this need not exclude adsorption.

All experiments (Fig. 2, 3 and 5; Table 2) affirm our initial assumption that deptropine can precipitate liver microsomes *in vitro*, thereby reducing the amount of enzymes required for *N*-demethylation (Fig. 1) and NADPH-oxidation (Fig. 4) in the reaction medium.

A number of structurally related compounds were investigated to test their possible effect on the stability of rat liver microsome suspensions (see Table 3).

The microsome-precipitating effect is strongest in the case of deptropine and BS 7039 (differing from deptropine only in having a —CH=CH— instead of a —CH_2—CH_2— bridge between the two phenyl groups), followed by 2-methylbenztropine and BS 7042. The other compounds had little or no effect. The experiments show

that an aromatic structure of the benzhydryl type, linked to a tropine ring, preferably by an oxygen atom, promotes liver microsome precipitation.

We found, moreover, that after centrifugation at 90,000 *g* the amount of microsomes recovered in the sediment was not at all quantitative (see Table 3). In addition, the ratio $N_{sediment}/N_{suspension}$ appeared to decrease with increasing microsome-precipitation, so that there were still unprecipitated microsomes after one hour's centrifuging.

TABLE 3. THE EFFECT OF DEPTROPINE AND RELATED COMPOUNDS, CONCENTRATION $5 \cdot 10^{-4}$ M, ON THE STABILITY OF RAT LIVER MICROSOMAL SUSPENSIONS

Exp.	Compound	mg N in supernatant*	mg N in sediment†	% mg $N_{sed.}$/mg $N_{sup.}$
1	—	1·480	0·872	59
	BS 6762	1·525	0·858	56
	BS 7051	1·415	0·812	57
	BS 7369	1·340	0·852	64
	Deptropine	0·555	0·175	31
2	—	1·350	0·946	70
	BS 6763	1·315	0·972	74
	BS 7039	0·295	0·142	48
	BS 7301	1·305	1·000	77
	Deptropine	0·395	0·130	33
3	—	1·700	0·946	56
	BS 7006	1·710	0·924	54
	BS 7042	1·370	0·702	52
	Deptropine	0·860	0·282	33
4	—	1·615	1·078	67
	2-Methylbenz-tropine	0·830	0·362	44
	Imipramine	1·530	0·886	58
	Atropine	1·725	1·030	60
	Deptropine	0·650	0·244	38

* A mixture of 9 ml of a rat liver microsomal suspension (see Materials and Methods) and 1 ml of a solution of the compound to be investigated in 0·1 M phosphate buffer (pH 7·4) was left overnight at 0° and centrifuged at 2000 *g*. The amount of nitrogen in the supernatant was determined by a micro Kjeldahl procedure.

† The supernatant was centrifuged at 90,000 *g* for one hr. The amount of nitrogen in the so obtained sediment was determined by a micro Kjeldahl procedure.

In vivo *demethylation of deptropine by rats*

The results of *in vivo* demethylation experiments (Table 4) with deptropine ($N-^{14}CH_3$) in rats show that, in contrast to what is found *in vitro*, deptropine is fairly rapidly demethylated *in vivo*: after 6 hr an average of 22·6 per cent of the administered radioactive dose is recovered in the expired air. After the same interval following pretreatment with SKF 525-A, this percentage is only 11·5, which means an inhibition of 50 per cent.

Similar experiments have been carried out with labeled nortriptyline by McMahon *et al.*[17] These authors also recovered 21% of the administered radioactive dose in the expiratory air after 6 hr and established a 50 per cent inhibition of the demethylation by SKF 525-A. *In vitro* nortriptyline is poorly demethylated.[17, 18] It might have the same pattern of association with and precipitation of liver microsomes,

as described for deptropine and related compounds. Nortriptyline has the same dibenzocycloheptene structure as deptropine.

McMahon[18] has also carried out combination experiments with amitriptyline and nortriptyline. In the author's opinion the observed inhibition by nortriptyline of the amitriptyline demethylation is due to the circumstance that nortriptyline binds

TABLE 4. *IN VIVO* DEMETHYLATION OF DEPTROPINE (—N—$^{14}CH_3$) IN RATS

Rat no.	Weight g	Percentage of administered radioactive dose in expiratory air						
		0–1 hr	1–2 hr	2–3 hr	3–4 hr	4–5 hr	5–6 hr	Total
1	190	5·6	4·5	2·9	2·4	1·8	1·5	18·7
2	152	10·2	7·8	2·5	2·2	1·5	1·1	25·3
3	193	8·8	7·2	3·0	2·1	1·4	1·3	23·8
4*	185	1·9	3·1	2·7	1·5	1·2	0·9	11·3
5*	169	3·2	2·6	2·0	1·8	1·0	1·1	11·7

* Pretreated with SKF 525-A.

preferentially with enzymes. However, in view of the fact that our combination experiments with diphenhydramine and the microsome-precipitating drug deptropine give the same picture, a nortriptyline-induced microsome precipitation must not be excluded as a cause for the inhibition.

Acknowledgements—The authors are indebted to Prof. Dr. H. K. Oosterhuis, Laboratory for Chemical Physiology, Vrije Universiteit, Amsterdam, for valuable advice during this investigation, to Smith Kline and French Laboratories for a sample of SKF 525-A and to Merck, Sharp and Dohme Nederland, N.V. for a sample of benztropine methane sulfonate.

REFERENCES

1. C. VAN DER STELT, A. F. HARMS and W. TH. NAUTA, *J. Med. Pharm. Chem.* **4**, 335 (1961).
2. A. B. H. FUNCKE, D. MULDER, M. C. DE JONGE, H. M. TERSTEEGE, R. DONNER and CHR. L RÜMKE, Arch. int. Pharmacodyn. **148**, 135 (1964).
3. W. HESPE, A. M. DE ROOS and W. TH. NAUTA, to be published.
4. J. AXELROD, *J. Pharmacol.* **114**, 430 (1955).
5. B. B. BRODIE, J. AXELROD, J. R. COOPER, L. GAUDETTE, B. N. LA DU, C. MITOMA and S. UDENFRIEND, *Science* **121**, 603 (1955).
6. B. N. LA DU, L. GAUDETTE, N. TROUSOF and B. B. BRODIE, *J. Biol. Chem.* **214**, 741 (1955).
7. B. B. BRODIE, J. R. GILLETTE and B. N. LA DU, *Ann. Rev. Biochem.* **27**, 427 (1958).
8. R. E. MCMAHON, *J. Med. Pharm. Chem.* **4**, 67 (1961).
9. R. E. MCMAHON and N. R. EASTON, *J. Pharmacol. exp. Ther.* **135**, 128 (1962).
10. L. J. ROTH, E. LEIFER, J. R. HOGNESS and W. H. LANGHAM, *J. Biol. Chem.* **176**, 249 (1948).
11. G. A. EDWARDS, C. H. EDWARDS and E. L. GADSDEN, *Int. J. appl. Radiation Isotopes* **4**, 264 (1959).
12. H. JEFFAY and J. ALVAREZ, *Analyt. Chem.* **33**, 612 (1961).
13. L. A. BAILLIE, *Int. J. appl. Radiation Isotopes* **8**, 1 (1960).
14. E. T. BUSH, *Analyt. Chem.* **35**, 1024 (1963).
15. J. R. GILLETTE, B. B. BRODIE and B. N. LA DU, *J. Pharmacol. exp. Ther.* **119**, 532 (1957).
16. J. FREUNDLICH, in: *Kapillarchemie*, 4th ed., Akademische Verlagsgesellschaft, Leipzig, 1932.
17. R. E. MCMAHON, F. J. MARSHALL, H. W. CULP and M. MILLER, *Biochem. Pharmacol.* **12**, 1207 (1963).
18. R. E. MCMAHON, *Life Sci.* **3**, 235 (1964).
19. H. MATTENHEIMER, in: *Mikromethoden für das klinisch-chemische Laboratorium*, p. 47, W. de Gruyter und Co., 1961.
20. M. J. JOHNSON, *J. Biol. Chem.* **181**, 707 (1949).