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INHIBITION OF BRAIN EPINEPHRINE SYNTHESIS BY 3,4-DICHLOROPHENYLETHANOLAMINE, A COMPETITIVE SUBSTRATE FOR NOREPINEPHRINE *N*-METHYLTRANSFERASE

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Abstract—3,4-Dichlorophenylethanolamine (DCPE) is a substrate for norepinephrine *N*-methyltransferase (NMT) from rat brain stem or from rabbit adrenal glands and competitively inhibits the methylation of (−)norepinephrine by NMT *in vitro*. The K_i value for inhibition of rat brain NMT by DCPE was 6×10^{-5} M. When DCPE was injected i.p. into rats at a dose of 50 mg/kg, epinephrine concentration in hypothalamus was reduced, although NMT activity measured in hypothalamic homogenates *in vitro* was not inhibited. The findings contrast in several ways to those with 8,9-dichloro-2,3,4,5-tetrahydro-1*H*-2-benzazepine (LY134046), a competitive inhibitor of rat brain NMT with an *in vitro* K_i of 2.4×10^{-8} M. Although the potency of DCPE was 1/2500th that of LY134046 *in vitro*, it lowered hypothalamic epinephrine concentrations at similar doses *in vivo*. The NMT activity measured *in vitro* in homogenates of hypothalamic tissue from LY134046-treated rats was reduced by a greater percentage than was epinephrine concentration. The reduction of epinephrine concentration *in vivo* by DCPE may relate to its ability to be a substrate for NMT, which would result in conversion of *S*-adenosylmethionine (SAMe) to *S*-adenosylhomocysteine (SAH) in NMT-containing neurons. The resulting increase in the SAH/SAMe ratio may inhibit norepinephrine *N*-methylation, since SAH is a competitive inhibitor of NMT when SAMe is the variable substrate. This mechanism is similar to that suggested for the decrease in epinephrine concentration in rat brain following L-dopa injection. L-Dopa injection increases SAH and decreases SAMe concentration measured in hypothalamus, due presumably to extensive *O*-methylation of dopa and its decarboxylated metabolites. In contrast, SAH and SAMe concentrations in hypothalamus were not measurably altered after DCPE injection, a not-unexpected finding since SAH/SAMe changes would occur only in NMT-containing neurons, which make up a negligibly small percentage by weight of hypothalamic tissue. DCPE and other NMT substrates may be able to inhibit epinephrine synthesis *in vivo* as effectively as non-substrate, competitive inhibitors having much higher affinity for NMT because their *N*-methylation elevates SAH/SAMe ratios specifically within NMT-containing neurons.

Inhibitors of norepinephrine *N*-methyltransferase (NMT) (the epinephrine-forming enzyme, EC 2.1.1.28, often called phenylethanolamine *N*-methyltransferase and abbreviated PNMT) have been shown to decrease epinephrine concentration in rat brain regions [1–5]. Generally, these inhibitors have been compounds resembling methyl-accepting substrates for the enzyme but have themselves not been substrates. For instance, phenylethylamines lacking the β-hydroxyl group are not substrates as phenylethanolamines are but, instead, are competitive inhibitors of NMT [6]. Several phenylethylamines, benzylamines, and conformationally restricted analogues of these compounds, are NMT inhibitors both *in vitro* and *in vivo* [7].

Fewer studies of alternative substrates for NMT as inhibitors of epinephrine formation have been reported. Grunewald and his colleagues [8, 9] described a compound, cyclooctylethanolamine, that competitively inhibits NMT *in vitro* and decreases the hypothalamic concentrations of epinephrine in rat brain *in vivo* [10]. We are reporting here some experiments with another NMT substrate that decreases epinephrine concentration in rat brain *in vivo*; our studies suggest that it may act at least partly through a mechanism different from that of the non-substrate NMT inhibitors.

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MATERIALS AND METHODS

Male Wistar rats, weighing about 150 g, were purchased from Harlan Industries, Cumberland, IN, and caged in groups of five in a 24° room with lights on from 7:00 a.m. to 7:00 p.m. After treatment, rats were decapitated, and brains were quickly removed and dissected. Tissue samples were frozen on dry ice and then stored at −15° prior to analysis.

3,4-Dichlorophenylethanolamine (DCPE) hydrochloride was purchased from the Aldrich Chemical Co., Milwaukee, WI. 8,9-Dichloro-2,3,4,5-tetrahydro-1*H*-2-benzazepine hydrochloride (LY134046) and *N*-[2-(*o*-chlorophenoxy)-ethyl]cyclopropylamine (LY51641) were synthesized in the Lilly Research Laboratories. L-Dopa (L-3,4-dihydroxyphenylalanine) was purchased from Monsanto, St. Louis, MO.

NMT from rabbit adrenal glands was partially purified and assayed radiometrically as described

previously [11]. NMT from rat brain stem was partially purified by the same ammonium sulfate fractionation procedure. With (−)norepinephrine as the methyl-accepting substrate, brain NMT was assayed radiometrically using the techniques of Henry *et al.* [12] as described previously [1]. With DCPE as the methyl-accepting substrate, brain NMT was assayed by the methods used by Deguchi and Barchas [13] with phenylethanolamine as substrate. NMT activity in brain regions from treated rats was assayed with (−)norepinephrine as substrate in the supernatant fluid after centrifugation of tissue homogenates at 27,000 *g* for 30 min.

Tissues were homogenized in 10 vol. of 0.5 mM dithiothreitol, and a portion of the homogenate was centrifuged at 27,000 *g* for 30 min. The supernatant fluid was used to assay NMT activity. The remaining portion of the whole homogenate was mixed with an equal volume of 0.2 N trichloroacetic acid (TCA) to precipitate proteins, and the protein-free supernatant fluid after centrifugation was used to assay for catecholamines or *S*-adenosylmethionine (SAMe) and *S*-adenosylhomocysteine (SAH). When NMT activity was not to be assayed, tissues were sonicated in 10 vol. of 0.1 N TCA directly, and an aliquot of the protein-free supernatant fluid after centrifugation was used for assay of catecholamines, SAMe and SAH.

Epinephrine and other catecholamines were determined by liquid chromatography with electrochemical detection by our previously described method [1]. SAMe and SAH were assayed by liquid chromatography with u.v. absorbance detection. The tissue extract was applied to an SP-C25 Sephadex column for preliminary isolation of SAMe and SAH. The column containing the adsorbed compounds was washed with 2 ml of 0.05 M ammonium acetate (pH 2.8, 10% methanol). After washing, SAH was eluted with 1 ml of 0.1 M ammonium acetate (pH 3.5, 10% methanol). The eluates were applied to a Vydac SCX column (30–40 μm), consisting of a 0.3 × 5 cm guard column and a 0.3 × 15 cm main column at a temperature of 50°. For SAH, the mobile phase was 0.1 M ammonium acetate (pH 3.1, 10% methanol), and for SAMe the mobile phase was 0.2 M ammonium acetate (pH 5.0, 10% methanol). The flow rate was 1 ml/min. Absorbance at 260 nm was monitored with an ISCO u.v. detector.

Tissue levels of DCPE were measured spectrofluorometrically after reaction with fluorescamine [14] by a method we have decribed previously [15].

All data from *in vivo* experiments are shown as mean values ± standard errors for five rats per group. Comparisons between groups were made by Student's *t*-test.

RESULTS

Inhibition of epinephrine formation by DCPE in vitro *and* in vivo. Figure 1 shows that DCPE inhibited rat brain stem NMT *in vitro* and that the inhibition was competitive with respect to (−)norepinephrine as the variable substrate. The highest concentration of inhibitor, 100 μM, produced inhibition ranging from 62 to 75% depending on the concentration of norepinephrine. Figure 1 shows that same data plotted as a Dixon plot. The point on the *x* axis, above which the four lines intersect, is the negative value for the K_i; from this graph the K_i for DCPE was determined to be 60 μM.

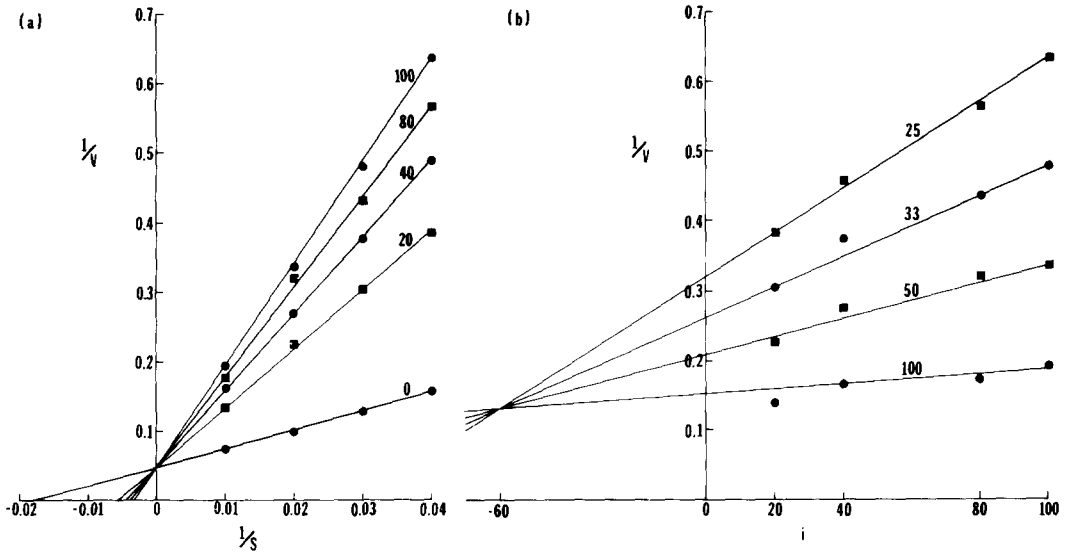

Fig. 1. Competitive inhibition of rat brain stem NMT by DCPE with (−)norepinephrine as the variable substrate. (a) Lineweaver–Burk plot showing competitive kinetics. The reciprocal of velocity (v) in pmoles of epinephrine formed per hr is plotted against the reciprocal of the micromolar concentration of (−)norepinephrine. The micromolar concentration of DCPE is shown above each line. *S*-Adenosyl-L-methionine concentration was 50 μM. (b) Dixon plot from which the K_i value for DCPE was determined from the point of intersection of the lines to be 60 μM. The reciprocal of velocity (v) in pmoles of epinephrine formed per hr is plotted against the micromolar concentration of the inhibitor, DCPE (i). The micromolar concentration of (−)norepinephrine is shown above each line. *S*-Adenosyl-L-methionine concentration was 50 μM.

Table 1. Effect of DCPE on catecholamine concentration in rat brain regions*

Treatment group	Catecholamines (pmoles/g)		
	Epinephrine	Norepinephrine	Dopamine
	Hypothalamus		
Control	66 ± 2	6106 ± 359	1075 ± 63
DCPE	46 ± 4† (−29%)	6216 ± 379	1109 ± 24
	Brain stem		
Control	21 ± 2	3129 ± 116	210 ± 8
DCPE	14 ± 1† (−33%)	2889 ± 102	217 ± 8

* DCPE (3,4-dichlorophenylethanolamine) hydrochloride was injected i.p. at 50 mg/kg 3 hr before rats were killed. Percentage changes are shown for groups that differed significantly from control.

† Different from control group ($P < 0.05$).

Table 1 shows catecholamine concentrations measured in hypothalamus and brain stem of rats treated with DCPE. Epinephrine concentration both in hypothalamus and in brain stem was reduced significantly. In contrast, neither norepinephrine nor dopamine concentration was altered significantly in either brain region. In this experiment, NMT activity was measured *in vitro* in homogenates of hypothalamus and found not to be measurably altered after DCPE injection (data not shown).

Table 2 shows the effect of DCPE on the increase in hypothalamic epinephrine concentration resulting from inhibition of monoamine oxidase. The increase is often taken as an indication of the rate of synthesis of a monoamine. In this experiment, epinephrine concentration, after DCPE alone, had returned to a point not significantly different from control due to the long time interval (6 hr). However, DCPE completely prevented the accumulation of epinephrine induced by the monoamine oxidase inhibitor, LY51641, indicating that epinephrine synthesis had been inhibited during most of the 6-hr period. In contrast, the increase in norepinephrine concentration and in dopamine concentration after LY51641 was not influenced significantly by DCPE, suggesting that the rates of synthesis of these two catecholamines were not affected.

Brain levels of DCPE. Figure 2 shows the concentration of DCPE measured in hypothalamus at various times after its i.p. injection and the concentration of endogenous epinephrine. Drug levels were readily measurable within 30 min and peaked at 1 hr after DCPE injection. Epinephrine concentration correlated inversely with DCPE concentration, being reduced most at 1 hr, the time of peak levels of DCPE in brain, and having returned to control levels by 4 hr, when DCPE had essentially disappeared.

Experiments pertaining to the mechanism of epinephrine lowering by DCPE in vivo. The effects of DCPE and of a non-substrate inhibitor of NMT are compared in Table 3. At 1 and 2 hr after the injection of DCPE, epinephrine concentration was decreased significantly. NMT activity, on the other hand, was not decreased at either time point. LY134046 at a 10 mg/kg dose caused a similar degree of depletion of epinephrine concentration, and NMT activity was reduced markedly at both 1 and 2 hr.

The concentrations of SAMe and SAH measured in rat hypothalamus 1 hr after the injection of DCPE or of L-dopa into rats are shown in Table 4. No significant alteration of either SAMe or SAH was found after DCPE, whereas L-dopa reduced SAMe concentration by half and increased SAH concentration 3-fold.

Table 2. Prevention by DCPE of the monoamine oxidase inhibitor-induced accumulation of hypothalamic catecholamines*

Treatment group	Catecholamines (pmoles/g hypothalamus)		
	Epinephrine	Norepinephrine	Dopamine
Control	90 ± 8	6,326 ± 286	1,049 ± 31
DCPE	72 ± 9	6,319 ± 480	1,074 ± 54
LY51641	121 ± 9† (+36%)	10,296 ± 261† (+63%)	1,819 ± 105† (+73%)
LY51641 + DCPE	92 ± 7‡	9,878 ± 229† (+56%)	1,591 ± 73† (+52%)

* DCPE hydrochloride (50 mg/kg, i.p.) and the monoamine oxidase inhibitor LY51641 (30 mg/kg, i.p.) were injected 6 hr before the rats were killed. Percentage changes are shown for all groups that differed significantly from control.

† Different from control group ($P < 0.05$).

‡ Different from LY51641 group ($P < 0.05$).

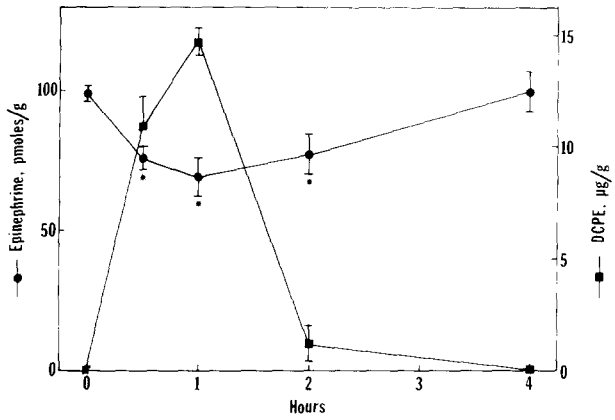

Fig. 2. Brain concentrations of DCPE and epinephrine as a function of time after DCPE injection. DCPE hydrochloride (50 mg/kg) was injected i.p. at zero times. Asterisks indicate significant decreases in epinephrine concentration ($P < 0.05$). Values are means ± S.E.; $N = 5$.

Table 3. Comparison of the effects of DCPE and of LY134046 (8,9-dichloro-2,3,4,5-tetrahydro-1*H*-2-benzazepine) on epinephrine concentration and NMT activity in rat hypothalamus*

Treatment group	Time (hr)	Epinephrine (pmoles/g)	NMT activity (pmoles/hr/g)
Control		104 ± 5	584 ± 20
DCPE	1	80 ± 6† (−23%)	596 ± 34
	2	86 ± 7† (−17%)	666 ± 18
LY134046	1	88 ± 2† (−16%)	226 ± 10† (−61%)
	2	86 ± 2† (−17%)	337 ± 31† (−42%)

* DCPE hydrochloride (50 mg/kg) and LY134046 (10 mg/kg) were injected i.p. Percentage changes are shown for groups that differed significantly from controls.
† Different from control ($P < 0.05$).

The substrate activity of DCPE is compared to that of norepinephrine in Fig. 3. With rat brain stem NMT as the enzyme, DCPE was methylated much more rapidly than (−)norepinephrine at low concentrations (Fig. 3a). Maximum substrate activity with the former compound occurred at 10^{-5} M, and the rate of methylation declined as the concentration was increased. The maximum rate of methylation of (−)norepinephrine occurred at 10^{-4} M, then substrate activity declined as the concentration was increased further. The maximum observed rate of methylation was greater with DCPE than with (−)norepinephrine. The methylation of DCPE was inhibited by SAH, a general inhibitor of methyltransferring enzymes (Fig. 3b). Even more potent as an inhibitor was LY134046, previously described as a potent NMT inhibitor [5]. Concentrations of LY134046 less than 10^{-6} M inhibited by more than

Table 4. Concentrations of *S*-adenosylmethionine and *S*-adenosylhomocysteine in rat hypothalamus after the injection of DCPE or L-dopa into rats*

Treatment group	Concentration (nmoles/g) *S*-Adenosylmethionine	*S*-Adenosylhomocysteine
(1) Vehicle	36.6 ± 0.9	2.2 ± 0.1
DCPE	37.5 ± 0.9	2.3 ± 0.1
(2) Vehicle	40.7 ± 1.5	1.4 ± 0.3
L-Dopa	20.2 ± 3.4†	4.6 ± 1.6†

* DCPE hydrochloride (50 mg/kg) or L-dopa methyl ester (200 mg/kg) was injected i.p. 1 hr before the rats were killed.
† Significant change ($P < 0.05$).

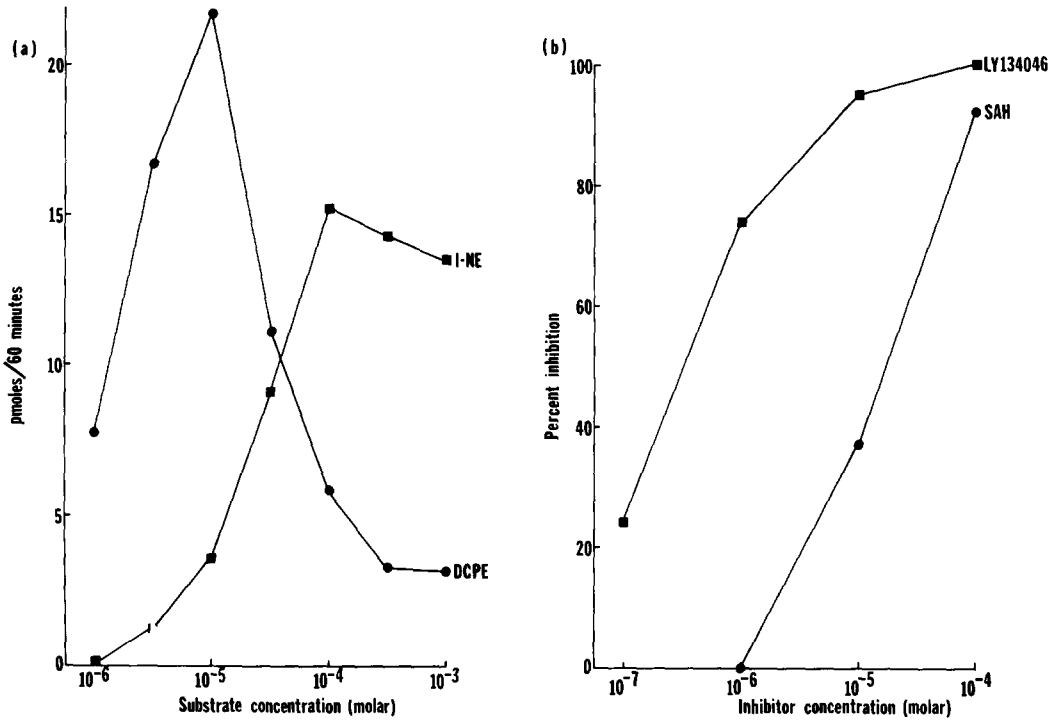

Fig. 3. (a) Comparative rates of methylation of DCPE (●) and of (−)norepinephrine (■) by rat brain NMT *in vitro*. The velocity of methylation, in units of pmoles/hr, is plotted against the molar concentration of each methyl-accepting substrate, the latter on a logarithmic scale. *S*-Adenosyl-L-methionine concentration was 50 μM. (b) Inhibition of DCPE methylation by LY134046 (■) and SAH (●). Percentage inhibition is plotted against the molar concentration of the inhibitor, the latter on a logarithmic scale. The concentration of DCPE was 10^{-5} M.

50% the methylation of DCPE present at 10^{-5} M. The potent inhibition by LY134046 supports the idea that NMT is the enzyme responsible for DCPE methylation.

DISCUSSION

The experiments described here reveal that DCPE is a substrate for rat brain stem NMT, as had been reported previously for rabbit adrenal NMT [6, 16]. The affinity of DCPE for rat brain NMT resulted in its being a competitive inhibitor when (−)norepinephrine was the variable substrate *in vitro*.

The affinity for NMT was similar with DCPE (K_i = 60 μM) as with (−)norepinephrine (K_m = 58 μM), which might suggest that DCPE would be effective in competitively inhibiting norepinephrine methylation by NMT *in vivo*. DCPE injected at a dose of 50 mg/kg, i.p., into rats significantly reduced epinephrine concentration in hypothalamus and brain stem. The lack of change in norepinephrine or dopamine concentration in either brain region favors the interpretation that the concentration of epinephrine decreased because of NMT inhibition. Comparison of DCPE with a previously reported competitive inhibitor of NMT, LY134046, revealed some differences between the two compounds. The K_i values of these two compounds as inhibitors of rat brain NMT *in vitro* were 2.4×10^{-8} M for LY134046 [5] and 6.0×10^{-5} M for DCPE (Fig. 1). Although the former compound is 2500 times more potent than the latter *in vitro*, the degree of epinephrine depletion by the two compounds was similar when doses that differed only 5-fold were given *in vivo* (Table 3). This fact immediately raises the possibility that DCPE might have some action *in vivo* in addition to simple competition with norepinephrine for the active site on NMT.

The fact that LY134046 caused a greater percentage reduction in NMT activity than in epinephrine concentration *in vivo* further supports that possibility, since DCPE in the same experiment caused no inhibition of NMT detectable by *in vitro* assay of the enzyme, whereas it lowered epinephrine concentration to the same extent as LY134046. Since these are competitive, reversible inhibitors of the enzyme, the argument can be made that assaying enzyme activity of hypothalamic homogenates *in vitro* may not reflect accurately the percentage inhibition of the enzyme *in vivo*, due to dilution that occurs during homogenization of the brain tissue and assay of enzyme activity. However, if the two compounds act by similar mechanisms, the degree of NMT inhibition as measured by *in vitro* assay of homogenates from treated rats should be similar at doses that produce similar decreases in epinephrine concentration.

If DCPE does not inhibit epinephrine synthesis *in vitro* by simple competitive inhibition of NMT, what alternative mechanism might be proposed? Recently, we have presented evidence that the ability of L-dopa to decrease epinephrine concentration in rat hypothalamus and to prevent the accumulation

of epinephrine following monoamine oxidase inhibition may result from the elevation of the SAH/SAMe ratio in rat hypothalamus following L-dopa injection [17, 18]. This elevation apparently results from extensive *O*-methylation of L-dopa and its metabolites by catechol *O*-methyltransferase (COMT; EC 2.1.1.6). The conversion of SAMe to SAH by this process results in a decrease in SAMe concentration and an increase in SAH concentration in hypothalamus [18]. Since SAH is a potent inhibitor of rat brain NMT [13] which acts competitively with SAMe as the variable substrate, the increased SAH/SAMe ratio would be expected to inhibit the conversion of norepinephrine to epinephrine by NMT and perhaps other transmethylation reactions as well. We have proposed that the decrease in epinephrine concentration following L-dopa injection [19] occurs through this mechanism [18].

The decrease in epinephrine concentration following DCPE injection may occur through a similar mechanism. The ability of DCPE to be a substrate for NMT could result in extensive conversion of SAMe to SAH in NMT-containing neurons following DCPE injection, whereas the *O*-methylation of L-dopa and its metabolites would occur in many different cells in brain and elsewhere due to the relatively ubiquitous distribution of COMT. COMT is present in all brain regions [20] and is not confined to catecholamine neurons, since destruction of these neurons with 6-hydroxydopamine does not decrease COMT in hypothalamus [21]. The methylation of DCPE would occur only in NMT-containing neurons, whose terminals make up only a tiny percentage of hypothalamic tissue [22]. Although an increase in the SAH/SAMe ratio after L-dopa injection would be expected to be, and is, readily measured in hypothalamus, the proposed increase in SAH/SAMe discretely within NMT-containing nerve terminals after DCPE injection would not be expected to lead to a measurable change in the SAH/SAMe ratio in total hypothalamus. Thus, the lack of change in hypothalamic SAMe and SAH concentrations in DCPE-treated rats is not unexpected.

Although direct proof is lacking, we propose that DCPE may reduce epinephrine biosynthesis secondary to increasing the concentration ratio of SAH/SAMe in NMT-containing neurons.

REFERENCES

1. R. W. Fuller and K. W. Perry, *Biochem. Pharmac.* **26**, 2087 (1977).
2. A. M. Sauter, J. Y. Lew, Y. Baba and M. Goldstein, *Life Sci.* **21**, 261 (1977).
3. J. M. Saavedra, *Brain Res.* **166**, 283 (1979).
4. I. N. Mefford, K. A. Roth and J. D. Barchas, *Neurosci. Lett.* **20**, 67 (1980).
5. R. W. Fuller, S. Hemrick-Luecke, R. E. Toomey, J-S. Horng, R. R. Ruffolo, Jr. and B. B. Molloy, *Biochem. Pharmac.* **30**, 1345 (1981).
6. R. W. Fuller, B. W. Roush and B. B. Molloy, *Adv. Enzyme Regulat.* **12**, 311 (1974).
7. R. W. Fuller, in *Transmethylation* (Eds. E. Usdin, R. T. Borchardt and C. R. Creveling), p. 251. Elsevier/North Holland, New York (1979).
8. G. L. Grunewald, J. M. Grindel, W. C. Vincek and R. T. Borchardt, *Molec. Pharmac.* **11**, 694 (1975).
9. W. C. Vincek, C. S. Aldrich, R. T. Borchardt and G. L. Grunewald, *J. med. Chem.* **24**, 7 (1981).
10. N. Y. Liang, R. E. Tessel, G. Grunewald and R. T. Borchardt, *Fedn Proc.* **40**, 725 (1981).
11. R. W. Fuller and J. M. Hunt, *Analyt. Biochem.* **16**, 349 (1966).
12. D. P. Henry, B. J. Starman, D. G. Johnson and R. H. Williams, *Life Sci.* **16**, 375 (1974).
13. T. Deguchi and J. Barchas, *J. biol. Chem.* **246**, 3175 (1971).
14. S. Udenfriend, S. Stein, P. Bohlen, W. Dairman, W. Leimgruber and M. Weigele, *Science* **178**, 871 (1972).
15. R. W. Fuller, K. W. Perry, J. C. Baker, C. J. Parli, N. Lee, W. A. Day and B. B. Molloy, *Biochem. Pharmac.* **23**, 3267 (1974).
16. R. W. Fuller, B. J. Warren and B. B. Molloy, *Biochim. biophys. Acta* **222**, 210 (1970).
17. R. W. Fuller and S. K. Hemrick-Luecke, *Biochem. Pharmac.* **30**, 3023 (1981).
18. R. W. Fuller, S. K. Hemrick-Luecke and K. W. Perry, *Fedn. Proc.* **41**, 1764 (1982).
19. A. Sauter and C. Suter, *Int. Soc. Neurochem. Abstr.* **8**, 227 (1981).
20. J. Broch, Jr. and F. Fonnum, *J. Neurochem.* **19**, 2049 (1972).
21. N. J. Uretsky and L. L. Iversen, *J. Neurochem.* **17**, 269 (1970).
22. T. Hokfelt, K. Fuxe, M. Goldstein and O. Johansson, *Brain Res.* **66**, 235 (1974).