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STUDIES ON THE MECHANISM OF PHENYLETHANOLAMINE-*N*-METHYL-TRANSFERASE INHIBITION BY A DICHLORO-SUBSTITUTED BENZIMIDAZOLE

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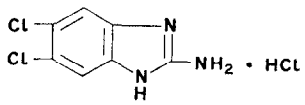
Abstract—2-Amino-5-6,-dichlorobenzimidazole (DCB) is the most potent inhibitor *in vitro* of phenylethanolamine-*N*-methyl-transferase (PNMT) known; in our laboratory its I_{50} for inhibiting a partially purified rabbit adrenal enzyme preparation with norepinephrine and *S*-adenosylmethionine as co-substrates was 4×10^{-7} M. DCB was not a substrate for PNMT at concentrations encompassing its inhibitory range (10^{-4}–10^{-8}M) and its antagonistic effect upon the enzyme after 30 min of preincubation was reversible. Kinetically, DCB was a noncompetitive inhibitor of norepinephrine and antagonized *S*-adenosylmethionine uncompetitively. Thus, the inhibition produced by this compound was not surmountable by either substrate. In kinetic studies at 1×10^{-4} M, epinephrine behaved as did DCB, which demonstrates a similarity in the mode of inhibitory action of the two compounds.

PHENYLETHANOLAMINE-*N*-methyl-transferase (PNMT) is an enzyme which catalyzes the terminal step in epinephrine biosynthesis, the transfer of a methyl group from *S*-adenosylmethionine (SAM) to the terminal nitrogen of norepinephrine (NE). As such, PNMT is highly localized in the adrenal medulla, although trace amounts have been reported in heart and brain.[1]

Several chemical classes of inhibitors of this enzyme have been described, including high concentrations of norepinephrine and epinephrine. With the exception of *S*-adenosylhomocysteine,[2] each of these inhibitor types is a structural analogue of norepinephrine; in fact, representatives of two of the classes, tranylcypromine and 5,6-dichloroamphetamine, have been reported to compete with norepinephrine (or normetanephrine) for its PNMT receptor site.[3,4] Although none of the known PNMT inhibitors has been conclusively shown to be effective *in vivo*, several potent antagonists *in vitro* have been described. The most potent agent in this regard is 5,6-dichloro-2-aminobenzimidazole (DCB), originally described by Mandel *et al.*[5] at the Merck Sharp & Dohme Laboratories.

One of the interesting aspects in the evaluation of a PNMT inhibitor is that of specificity, which in turn, is related to its mechanism of action. Particularly is this true with compounds which are competitive with norepinephrine, since an agent of this type may well inhibit other enzymes or receptors, or both, for which this catecholamine is a substrate or agonist. This report attempts to deal with this question, via an examination of: (1) the enzymatic mechanism of action of the above benzimidazole derivative in inhibiting PNMT, and (2) its effects in other test systems *in vitro*

in which norepinephrine is a physiological participant. The chemical structure of DCB is shown below:



DCB

METHODS

PNMT assay. A partially purified, lyophilized PNMT preparation derived from rabbit adrenals was obtained commercially from Gallard–Schlesinger Company. The enzyme was solubilized in potassium phosphate buffer and the reaction run in 300 μl constituted as follows: PNMT, 280 μg; phosphate buffer (pH 7·4), 50 μmoles; l-norepinephrine, 9 nmoles; and *S*-adenosylmethionine-^{14}C (SAM; ~ 20,000 dis./min), 9 nmoles. The label was localized on the reactive methyl group of the SAM molecule. The reaction was run for 30 min at 37° after which it was terminated with 1 N HCl (200 μl). Approximately 1 g of solid NaCl was then added and the solution extracted with 6 ml of acid-washed NaCl-saturated butanol. One ml of the butanol layer, containing labeled epinephrine, was then added to 10 ml of an aqueous BBOT phosphor, counted in a Tri-Carb liquid scintillation spectrophotometer for 10 min, and quantitated in terms of millimicromoles of epinephrine. A correction was made for the small amount of SAM (approximately 130 dis./min) coming through the extraction procedure. The radioactive reaction product extracted into the butanol phase was identified, after acetylation, as epinephrine, by using Silica gel thin-layer plates (Merck, F254) with a chloroform–acetic acid–2B-ethanol (95:5:5) solvent system.

Monoamine oxidase assay. Monoamine oxidase (MAO) was assayed using rat liver mitochondria, isolated according to the method of Green and Sawyer.[6] The particles were suspended in potassium phosphate buffer such that 1 ml of the suspension was equal to 10 mg of original wet weight (1 mg/100 μl). Enzyme preparation (100 μl) was then added to an incubation media containing 100 μl tryptamine-2-^{14}C (18 nmoles, ~ 11,000 dis./min) and 100 μl potassium phosphate buffer (pH 7·4, 50 μmoles). The reaction was run for 20 min at 37° after which it was stopped by the addition of 200 μl of 1 N HCl. The solution was then extracted with 6 ml toluene; 4 ml of the extract was subsequently added to 10 ml of BBOT phosphor and the ^{14}C content measured in a Tri-Carb liquid scintillation counter. In the reaction, labeled tryptamine is oxidized to the corresponding labeled aldehyde (identified chromatographically) which, unlike the parent compound, is readily extracted into toluene, thus making possible a quantitation of the reaction velocity. Results are expressed as millimicromoles of product (aldehyde) synthesized per assay period (20 min), after correction for the small amount of tryptamine (approximately 20 dis./min) in the toluene phase.

In both the above assays, the reaction rates were linear with respect to time and enzyme concentration and the substrate concentrations were near the peak of the substrate-dependent portion of the respective substrate–velocity curve. Each assay was performed in quadruplicate. Apparent K_m, V_{max} and K_i values were graphically determined by methods described by Mahler and Cordes[7] and Bray and White.[8]

Preparation of isolated rabbit aortic tissue. New Zealand white rabbits, weighing 2·0–2·5 kg, were sacrificed by cervical concussion and the thoracic aortae immediately excised. The aortae were then immersed in warm, oxygenated Krebs–bicarbonate solution and cut in 3 mm, 15°, right-handed spirals, according to the method of Furchgott and Bhadrakom.[9] The spirals were cut into 3-cm strips, each of which was suspended vertically in a 50-ml tissue bath containing isotonic Krebs–bicarbonate solution. The composition of this buffer was as follows (amount in millimoles per liter): NaCl, 142·4; KCl, 5·6; $CaCl_2$, 3·0; KH_2PO_4, 1·4; $NaHCO_3$, 29·8; dextrose, 12·0. The bathing medium was aerated with 95% O_2 –5%CO_2 and maintained at 37·5 $\pm$ 0·5° and pH 7·4. The lower end of each strip was secured to a tissue holder and the upper end attached via a length of fine surgical cotton to a Sanborn force-displacement transducer (FTA-3 or FTA-10); responses were displayed on two-channel Sanborn recorders (model 296). Tissues were placed under an initial tension of 4·0 g and permitted to stretch and equilibrate for 3·5 hr prior to testing. The tension on the strips was readjusted to 4·0 g every 30 min during the equilibration period by elevating the transducer levels. Drugs were introduced into the tissue baths with microsyringes or pipettes and removed by overflow and replacement of the bathing medium from preheated, oxygenated reservoirs; a total of three bath volumes (150 ml) was used for each wash. Responses were expressed as gram changes in tension above baseline tone.

In testing for tissue stimulatory (α-agonist) activity, DCB was pipetted into the bath in a cumulative manner with doses ranging from 2×10^{-6} to 1×10^{-3} M. In the absence of detectable response, each dose was given 10 min after the preceding one; when responses were obtained, subsequent doses were given at peak response. In testing DCB for norepinephrine antagonist activity (α-adrenergic blockade), a control dose–response curve to norepinephrine was first obtained in each strip in order to ascertain the maximal tissue response to this agonist. After recovery, the drug was incubated with the tissue for 10 min before an experimental, cumulative norepinephrine dose–response curve was begun. Responses obtained in each experimental curve were then expressed as a percentage of the maximal response in the initial control curve.

Statistical analysis of all data was by methods described in Snedecor.[10] All concentrations referred to in this report are final medium levels.

RESULTS

Inhibition of PNMT. A complete dose–response curve for the inhibition of PNMT by DCB under our standard assay conditions is shown in Fig. 1. The curve obtained in this study is classically sigmoid shaped and the I_{50} concentration is 4×10^{-7} M. The data depicted in Fig. 2 further demonstrate that the drug is not a substrate for the enzyme over the inhibitory concentration range described in Fig. 1. Thus, DCB is a potent inhibitor of the *N*-methylation of norepinephrine by PNMT, without itself functioning as a methyl group acceptor. The drug has a partition coefficient in our extraction procedure of 26. Since the *N*-methylated derivative of DCB should be even more lipid (butanol) soluble than the parent compound, it is unlikely that the result obtained was artificial due to non-extractability of methylated DCB.

One of the important considerations in analyzing the nature of an enzyme inhibition process is that of reversibility; i.e. is the inhibitor–enzyme complex dissociable when

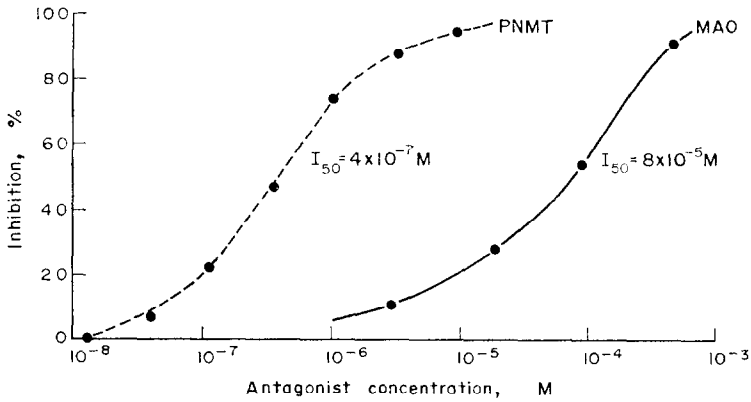


FIG. 1. Dose-response curves showing the effects of DCB as an inhibitor of PNMT and MAO. Each point is the mean of four values.

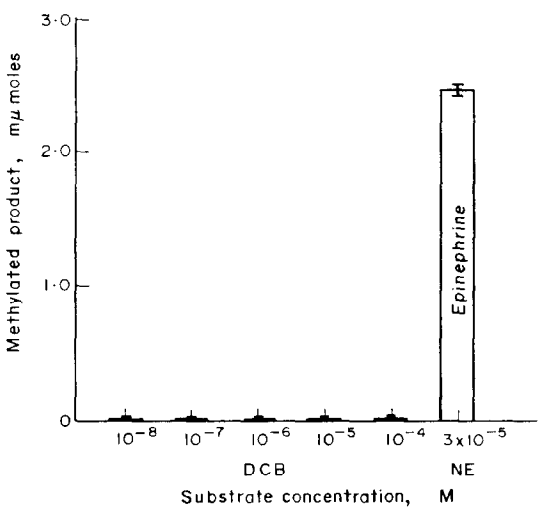


FIG. 2. Effect of DCB as a substrate for PNMT. Norepinephrine was run in the same assay at 3 × 10^{-5} M for comparative purposes and each assay was run for 30 min. All points are the mean of quadruplicate measurements. Bars indicate standard error of the mean.

the drug is removed from contact with the enzyme? Studies were performed in which the drug (3·75 × 10^{-6} M) was incubated for 30 min with a concentrated enzyme solution (640 μg/300 μl) which was then dialyzed for 24 hr, diluted and run under standard PNMT assay conditions. The final dilution was 3-fold. Under these conditions no significant inhibition was produced with the drug-pretreated and then dialyzed enzyme, indicating that the drug–enzyme complex is reversible (Table 1).

Kinetic studies. The kinetic studies performed with DCB are graphically shown in Figs. 3 and 4. The substrate–velocity curves show that the inhibition produced by this drug at two concentrations was not surmountable by either substrate, which rules out

TABLE 1. REVERSAL BY DIALYSIS OF PNMT INHIBITION BY 2-AMINO-5,6 DICHLOROBENZIMIDAZOLE

Conditions	PNMT activity	Inhibition (%)
(a) No inhibitor, and not dialyzed	2·55 ± 0·07	
(b) Inhibitor ($3{\cdot}75 \times 10^{-6}$ M) added at beginning but not dialyzed	0·42 ± 0·06	84
(c) No inhibitor but dialyzed	2·30 ± 0·14	
(d) Inhibitor ($3{\cdot}75 \times 10^{-6}$ M) added at beginning and dialyzed	2·26 ± 0·02	2

Enzyme preps were incubated for 30 min at 37° in pH 7·4 phosphate buffer and then for 24 h at 4° prior to use as enzyme in PNMT assay. Mixtures (c) and (d) were dialyzed during this time against fequent changes of the buffer. Inhibitor was added before the initial incubation in (b) and (d). Inhibitor concentration was 3·75 μM in the dialysis mixtures and 1·25 μM in the final assay mixture. PNMT activity is in mμmoles of epinephrine formed per 30 min of incubation with 280 μg protein. Each experimental set was done in quadruplicate.

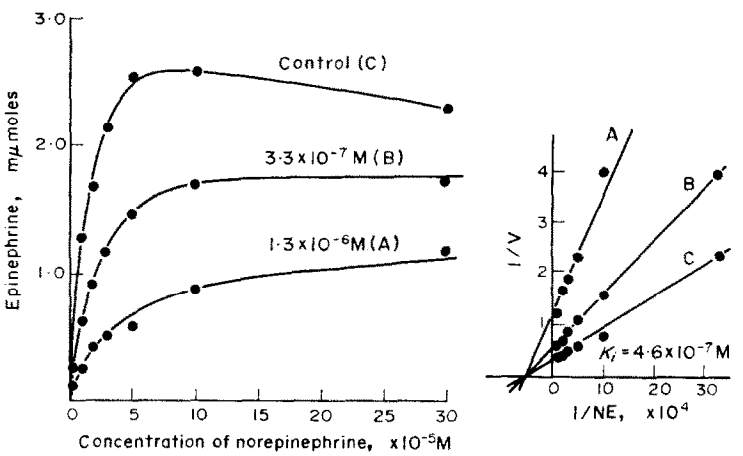


FIG. 3. Kinetic analysis of PNMT inhibition produced by DCB. Norepinephrine was the variable substrate in the experiment; the fixed concentration of SAM was 3×10^{-5} M. The reaction was run for 30 min and the velocity is expressed in terms of millimicromoles of epinephine formed per assay period. Concentrations listed above each curve are those of DCB.

a competitive mechanism of action. This conclusion is substantiated by Lineweaver–Burk plots of the data, which indicate that DCB inhibits the enzyme in a noncompetitive manner with respect to norepinephrine and uncompetitively with respect to *S*-adenosylmethionine.

It has been previously reported that epinephrine, the natural product of the PNMT-catalyzed reaction, is itself an inhibitor of the enzyme at high, although perhaps physiological, concentrations.[11] Interestingly, the kinetic picture of the inhibition produced by this hormone is similar to that seen with DCB, that is, noncompetitive

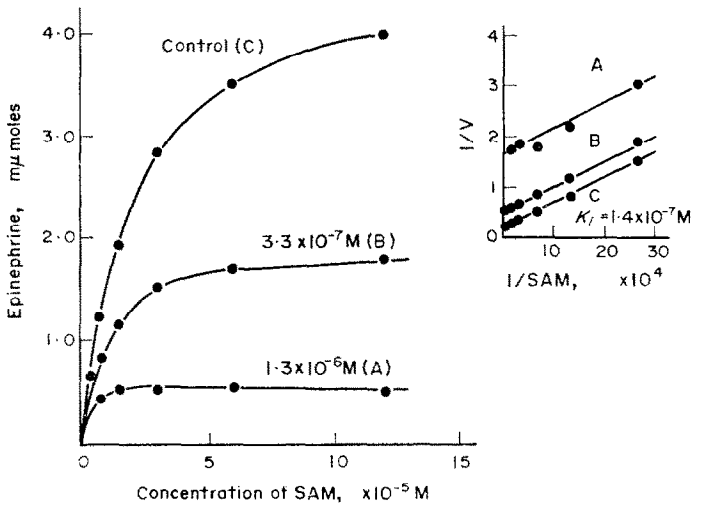


FIG. 4. Kinetic analysis of PNMT inhibition produced by DCB. SAM was the variable substrate in this experiment; the fixed concentration of NE was 3×10^{-5} M. The reaction was run for 30 min and the velocity shown is expressed in terms of millimicromoles of epinephrine formed per assay period. Concentrations listed above each curve are those of DCB.

with respect to norepinephrine and uncompetitive with regard to SAM (Figs. 5 and 6). Apparent K_i values for both epinephrine and DCB were calculated from linear replots of the primary kinetic data ($1/V_{max}$ vs. i)[12].

Biological specificity studies. The biological specificity of DCB was tested in systems *in vitro* which are similar to PNMT in that norepinephrine and related phenethylamines may function in them under physiological conditions as a substrate or receptor agonist. The first of these studies (Fig. 1) indicates that DCB is approximately 200

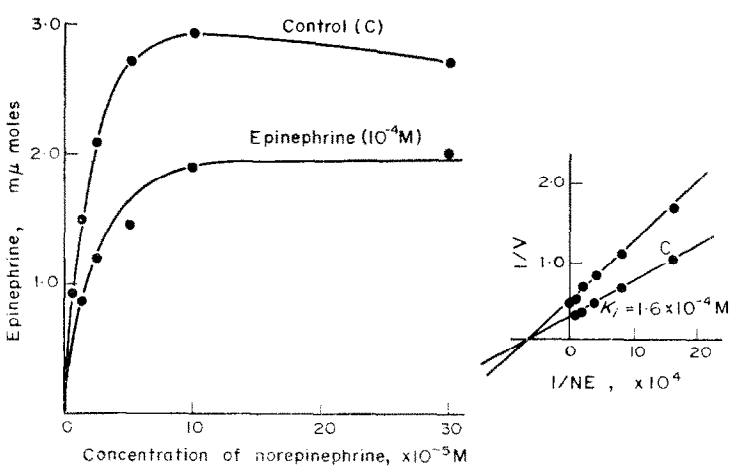


FIG. 5. Kinetic analysis of PNMT inhibition produced by epinephrine at a single concentration (1×10^{-4} M). Norepinephrine was the variable substrate in this experiment; the fixed concentration of SAM was 3×10^{-5} M. The reaction was run for 30 min and the velocity is expressed in terms of millimicromoles of epinephrine produced per assay period.

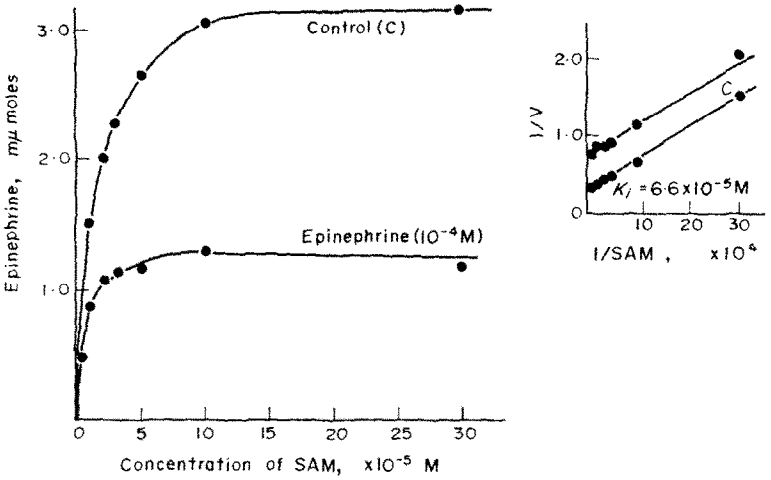


FIG. 6. Kinetic analysis of PNMT inhibition produced by epinephrine at a single concentration (1×10^{-4} M). SAM was the variable substrate in this experiment; the fixed concentration of norepinephrine was 3×10^{-5} M. The reaction was run for 30 min and the velocity is expressed in terms of millimicromoles of epinephrine produced per assay period.

times more potent as an inhibitor of PNMT than of MAO. These results are in direct contrast to data obtained with tranylcypromine in these assays showing that it is a much more potent inhibitor of MAO ($I_{50} = 2{\cdot}5 \times 10^{-6}$ M) than of PNMT ($I_{50} = 9{\cdot}0 \times 10^{-5}$ M).

The results obtained in Fig. 7 indicate that DCB is not an alpha-adrenergic blocking agent over, at least, the major part of its PNMT inhibitory concentration range. At

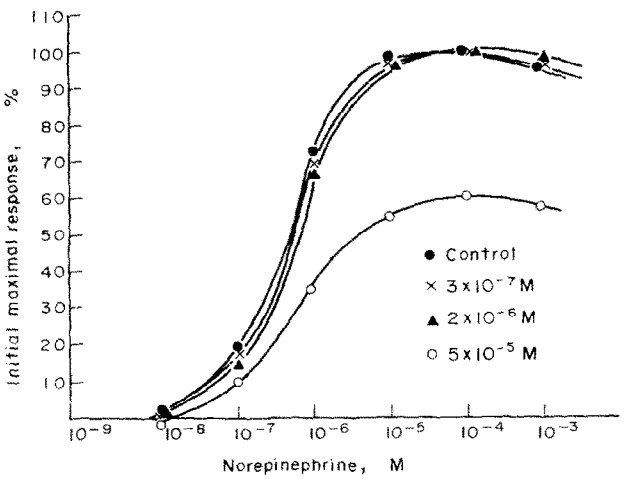


FIG. 7. Effect of DCB as an inhibitor of the norepinephrine-induced contraction of the rabbit aortic strip. Concentrations listed within figures are those of DCB. Each point shown represents the mean of three experiments.

5×10^{-5} M, however, the drug did partially antagonize the response of the tissue (rabbit aortic strip) to norepinephrine in a noncompetitive manner. Initial testing had demonstrated that the drug was devoid of significant intrinsic stimulating (including α-receptor agonist) activity upon this tissue at concentrations up to 1×10^{-3} M.

DISCUSSION

The data indicate that DCB is a very potent inhibitor *in vitro* of PNMT; in fact, it is the most potent compound we have tested to date. The compound is not a substrate for the enzyme and hence qualifies as a true inhibitor as opposed to a co-substrate. The results obtained in the dialysis experiments indicate that DCB is a reversible inhibitor of PNMT, which indicates that the drug–receptor complex is dissociable.

The outcome of the kinetic experiments with DCB was somewhat surprising in view of: (1) published reports indicating that tranylcypromine and 5,6-dichloroamphetamine are competitive inhibitors of norepinephrine (normetanephrine), and (2) the structural similarity of the compound to norepinephrine. Our results, however, show that DCB is a noncompetitive inhibitor with respect to norepinephrine as evidenced by the Lineweaver–Burk plot indicating that the V_{max} of the enzyme is reduced by the drug without altering the K_m when norepinephrine is the variable substrate. When SAM is employed as the variable substrate, the mode of inhibition produced by the drug is also not of a competitive nature, since the V_{max} of the enzyme is reduced in a dose-dependent fashion. The K_m, however, is also altered in this case and the Lineweaver–Burk reciprocal plots for the drug-treated reaction are parallel with the control, which is indicative of uncompetitive inhibition. Mechanistically, the prime difference between noncompetitive and uncompetitive inhibition is that in the latter the inhibitor and substrate do not bind independently to the enzyme. From an overall point of view, however, they are similar types of inhibition: (1) in not being completely reversible or surmountable by excess substrate; and (2) by the fact that the inhibitor does not occupy the same receptor site as the substrate, except in case where the drug is reacting irreversibly with the substrate site.[7]

Quantitatively, the relative lack of potency of DCB as an MAO inhibitor and an α-adrenergic antagonist shows that this compound has much less affinity for these receptor sites than for PNMT. Qualitatively, however, it is of interest to note that DCB showed a similar mode of action (noncompetitive) in inhibiting norepinephrine, both as an α-agonist and as a substrate for PNMT.

Epinephrine at 1×10^{-4} M was found to behave similarly to DCB as an inhibitor of PNMT, which suggests, but certainly does not prove, that the two compounds may be acting at the same inhibitory receptor site. Thus, the drug may be interacting with the enzyme at a site normally utilized as a feedback control by epinephrine of its own synthesis rate, rather than competing with norepinephrine at a substrate site.

Despite the potency of DCB *in vitro*, Mandel *et al.*[5] reported that it reduced only slightly the adrenal ratio of epinephrine/norepinephrine in mice after very high i.p. dosage. Similarly, we have found no effect of the compound on adrenal epinephrine levels in rats after parenteral administration (s.c.) for 14·5 days on a b.i.d. basis at doses up to 162 mg/kg/day (unpublished observation). This observation suggests that DCB is not active *in vivo* as a PNMT inhibitor, since the levels of epinephrine are markedly reduced when the activity of this enzyme is decreased after hypophysectomy.[13]

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