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EFFECTS OF THE TUMOR INHIBITOR, VERNOLEPIN, AND OF ITS THIOETHER ADDUCT, ON THE ACTIVITY OF CATECHOL *O*-METHYLTRANSFERASE *IN VITRO**

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Abstract—The activity of cat erythrocyte lysate catechol *O*-methyltransferase (COMT) *in vitro* is shown to be amenable to control by a variety of reagents including an SH reactive tumor inhibitor (vernolepin), its non-SH reactive derivative (the bis-*n*-propanethiol adduct of vernolepin), *n*-propanethiol itself, and the organic solvent, methanol. The ability of the adduct to modify COMT activity extends to all other sources examined (partially purified rat liver COMT and 78,000 *g* supernatant from rat liver, kidney, spleen and brain). Alterations in kinetic parameters (apparent K_m and V_{max}) were observed when the adduct of vernolepin enhanced cat erythrocyte COMT activity.

The sesquiterpene lactone, vernolepin, has been shown [1] to have tumor inhibitory activity against the Walker 256 intramuscular carcinosarcoma *in vivo*, as well as cytotoxic activity *in vitro* against cells derived from a carcinoma of the human nasopharynx (KB). This anti-tumor activity was specific for the SH reactive parent compound, vernolepin, since its bis-cysteine adduct was inactive against the KB cell line. Other tumor inhibitors of plant origin, namely eupatundin and elephantopin, have been converted to their mono-cysteine adducts with a decrease in potency as cytotoxic agents, while their bis-cysteine adducts were completely inactive. The bis-*n*-propanethiol adduct of vernolepin is no longer reactive toward thiol addition, and its biological activity would presumably be lost as well.

Chemically, the addition of vernolepin to several common biological nucleophiles has been studied [2], and it was found that the drug had a greater affinity for thiols than for other common biological nucleophiles such as amines. The addition of vernolepin to low molecular weight thiols could be reversed by a retro-Michael-type reaction involving chemical methylation of the thioether bond (with methyl iodide) followed by treatment with mild base (sodium bicarbonate).

This chemical work has been extended biologically by Hanson *et al.* [3] to show that the enzyme phosphofructokinase is inactivated *in vitro* by vernolepin coincident with the loss from the enzyme of SH groups. Smith *et al.* [4] have similarly shown that glycogen synthase becomes completely inactive after alkylation of three of its six sulfhydryl groups/90,000 Dalton subunit. The above work leads to a general hypothesis that the selectivity *in vivo* of such compounds as tumor inhibitors may relate to an enhanced uptake, or a decreased release, of the drug from its SH binding sites in tumor vs normal tissue. One specific mechanism [5] which might account for such differences could be the relative ease with which the thioether adducts are methylated to sulfonium salts (and then undergo thioether elimination) in normal vs neoplastic tissues.

While examining various tissue homogenates and erythrocyte lysates for an enzymatic function that could methylate the thioether bond of vernolepin adducts, we included both norepinephrine and adduct in some assay mixtures with the view that, should adduct serve as a substrate for catechol *O*-methyltransferase (COMT), we might observe a decrease in COMT activity toward norepinephrine. To the contrary, we observed enhanced production of ^{3}H-normetanephrine in these assays. In this paper, evidence is presented‖ that the SH reactive parent compound, vernolepin (Fig. 1), which previously was known only to inhibit the activity of sulfhydryl enzymes, may also enhance the activity of COMT under some conditions. In addition, a biological action (enhancement of COMT activity *in vitro*) has been observed for the first time for a non-SH reactive derivative of vernolepin (namely, its bis-*n*-propanethiol adduct, Fig. 2). Further, the activity of COMT *in vitro* is shown to be readily altered by several other reagents including *n*-propanethiol and the organic solvent, methanol.

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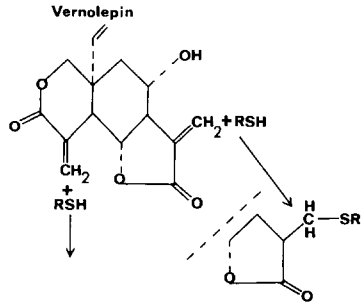

Fig. 1. Tumor inhibitor, vernolepin, reacts with thiols at the two α-methylene lactone sites indicated. When low molecular weight thiols (such as *n*-propanethiol or cysteine) are used, the reaction leads to formation of the thioether products of addition at both sites.

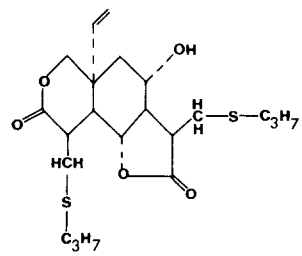

Fig. 2. The bis-*n*-propanethiol adduct of vernolepin.

MATERIALS AND METHODS

COMT was prepared from several sources as described below.

Cat red blood cell extracts. Heparinized blood [6] from phenobarbital anaesthetized mongrel cats was centrifuged at 2000 *g* at 4° for 10 min, and the plasma removed by aspiration. After lysis by freezing (liquid nitrogen, 2 min) and thawing (water bath, 37°, 3–5 min), 1 ml lysate was diluted with 9 ml distilled water (4°). The lysate was frozen at −70° in small aliquots which were thawed when needed (once) and added to reaction tubes containing buffer, substrate and other reagents as described below.

Rat liver, kidney, spleen and brain extracts. Adult male Wistar strain rats were killed by cervical dislocation; organs were rapidly removed and chilled on ice. All subsequent steps were carried out at 4°. Organs were homogenized in 4 vol. (w/v) 1·15% KCl, and the homogenates centrifuged at 78,000 *g* for 10 min. The 78,000 *g* supernatants were frozen at −70° and thawed once before use.

Partially purified rat liver COMT. The method of Axelrod [7] was used with the modifications indicated below. All steps were carried out at 4°. The 78,000 *g* supernatant prepared as above was acidified to pH 5·0 with dropwise addition of 1 N acetic acid. After standing for 5 min, the resultant precipitate was removed by centrifugation (27,000 *g*, 15 min) and to the supernatant (300 ml) was added 52 g ammonium sulfate (30 per cent saturation). After gentle stirring for 10 min, the precipitate was again removed by centrifugation as above, and an additional 34 g ammonium sulfate was added (50 per cent saturation). After standing for 10 min, the precipitate was collected by centrifugation as above and dissolved in 50 ml ice-cold distilled water. The solution was dialyzed against 0·001 M phosphate buffer, pH 7·0, for 6 hr. The concentration of protein was determined by a modification of the method of Lowry *et al.* [8]. The dialysate was diluted to 9 mg/ml of protein with 0·02 M acetate buffer, pH 5·0, and divided into ten 10-ml aliquots. To each aliquot was added 4·5 ml calcium phosphate gel (CalBiochem, grade A) containing from 10 to 50 mg/ml of solids. After gentle mixing for 15 min, each fraction was centrifuged at 2000 *g* for 5 min, and the supernatant was removed and frozen at −70°. The pellet was eluted twice with 2·5 ml of 0·02 M phosphate buffer, pH 6·9. The combined eluants were also stored at −70°. Both supernatants and eluants were assayed for COMT activity. In the experiment in Fig. 7, the source of enzyme was a supernatant from a 30 mg/ml calcium phosphate gel fraction. This enzyme had been purified approximately 100-fold as compared to the homogenate.

Vernolepin and its bis-adduct. The bis-*n*-propanethiol adduct of vernolepin was prepared as previously described [5]. Vernolepin, as well as its adduct, was dissolved in methanol giving stock solutions containing 0·6 μmole compound/10 μl of solution. The stock solutions were stored in a refrigerator freezer.

Methanol controls. The effector agents studied in these experiments (vernolepin and its bis-adduct) were quite water insoluble and were dissolved in methanol. Thus, it was necessary to determine the effect of this solvent alone on COMT activity. We observed early that methanol, at 1·3% (v/v), might either slightly increase or decrease enzyme activity, depending on the length of storage of the enzyme preparation and probably other, as yet undefined, factors. Therefore, a control consisting of norepinephrine plus methanol (vehicle) was included in each experiment. In this way, the effect of the solvent alone on COMT activity was established in each experiment, and the effect of a given perturbing reagent on enzyme activity was measured in comparison to this solvent effect.

Standard reaction mixtures. In a final volume of 745 μl were contained the following components: 50 μmoles phosphate buffer, pH 7·9; 10 μmoles $MgCl_2$; 0·3 μmole *l*-norepinephrine *d*-bitartrate (NE) in 0·001 N HCl or NE plus one of the following: methanol (1·3%, v/v), methanol containing 0·6 μmole vernolepin, methanol containing 0·6 μmole *n*-propanethiol, or methanol containing 0·6 μmole bis-*n*-propanethiol adduct of vernolepin (adduct); 0·58 μCi (72 pmoles) ^{3}H-*S*-adenosyl-L-methionine chloride (^{3}H-SAM, sp. act. 8·02 Ci/m-mole, New England Nuclear, Boston, Mass.); and 500 μl of the appropriate enzyme preparation.

Incubation and extraction. The method of Axelrod and Cohn [6] was followed. Reactions were started by pipetting 500 μl enzyme into charged reaction tubes, mixing and incubating at 37° in a water bath with intermittent shaking. At the appropriate time intervals, tubes were removed, placed in an ice bath, and 0·5 ml, pH 10, borate buffer (0·5 M) was rapidly added and the contents were mixed to stop the reaction. Three ml isoamyl alcohol was next added, the contents were again mixed and all tubes centrifuged at 2000 *g* for 5 min (4°). One-ml aliquots of the isoamyl extracts were transferred to scintillation vials

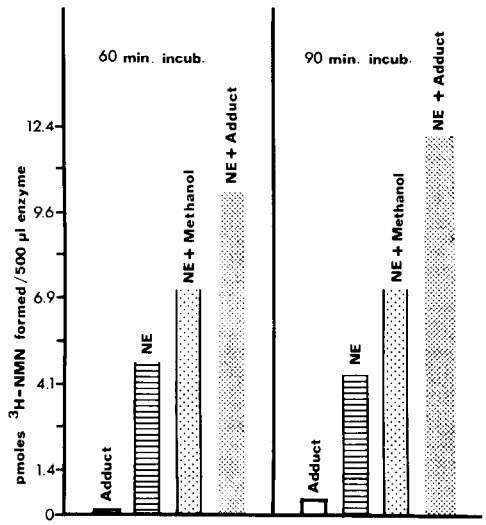

Fig. 3. Cat erythrocyte lysate COMT activity in a 60- or 90-min assay in the presence of the bis-*n*-propanethiol adduct of vernolepin (0·81 mM), norepinephrine (0·4 mM), norepinephrine plus methanol (1·3%, v/v), or norepinephrine plus methanol plus adduct (0·81 mM).

and evaporated to dryness in an evacuated chromatographic drying oven for 2 hr at 80° in a stream of warm air. After cooling, scintillation mixture* was added and radioactivity determined in a Beckman LS-230 scintillation counter. Counting efficiency was determined to be 38 per cent by use of ^{3}H-toluene as internal standard.

Enzyme activity. Enzyme activity is expressed as pmoles ^{3}H-normetanephrine formed/500 µl of enzyme/time period (usually 5 min). Exceptions are noted in legends for individual figures.

Thin layer chromatography (TLC). Fifty µl of the isoamyl alcohol extract was spotted on a cellulose thin-layer chromatography plate (0·1 mm thick, Cel 300–10 u.v.$_{254}$, Brinkman Instruments, Inc.). One of three solvent systems was used to develop plates and, after development (ascending), the plate was air dyed. Starting at the origin, 1-cm wide bands were scraped and the scrapings were placed in individual scintillation vials. Scintillation mixture was added and radioactivity determined as above.

RESULTS

The initial observation of enhanced cat erythrocyte lysate COMT activity by the adduct is shown in Fig. 3. In a 60-min assay, 1·3% (v/v) methanol caused a 48 per cent increase in activity when compared to the norepinephrine control, while addition of adduct to a final concentration of 0·81 mM resulted in a further 46 per cent increase in activity when compared to the methanol control. A still larger increase in activity to a level 80 per cent above the methanol control was observed in the presence of the adduct in a 90-min assay. In the presence of adduct alone (no exogenous norepinephrine added), only negligible activity was observed at 60 or 90 min of incubation. Table 1 demonstrates that the R_f of the radioactive product obtained from either norepinephrine alone or norepinephrine plus the adduct is identical to the R_f of authentic ^{3}H-normetanephrine in three solvent systems.

We next examined the molecular specificity of this effect. Figure 4 shows the activity of cat erythrocyte COMT at 60 min of incubation in the presence of the substrate, norepinephrine, and various other reagents. A slight but non-significant decrease in activity occurred in the presence of 1·3% methanol

Table 1. Thin-layer chromatography (cellulose support) of authentic ^{3}H-normethanephrine (^{3}H-NMN) in three solvent systems*

Solvent System	R_f of ^{3}H-Normetanephrine		
	Authentic	Enzymatic	
		NE	NE + Adduct
I EtOH/HOAc/H_2O 64 : 1 : 35	0.68	0.68	0.68
II n-BuOH/HOAc/H_2O 8 : 2 : 2	0.57	0.57	0.57
III n-BuOH/2-PrOH/NH_4OH/H_2O 3 : 1 : 1 : 1	0.54	0.54	0.54

* The enzymatic product was from the 60-min assay in Fig. 3. Starting at the origin, 1-cm wide bands were scraped and counted. Radioactivity isolated in a particular 1-cm band was assigned a distance midway in the band for purposes of R_f calculation. (Thus, radioactivity isolated in the band between 11 and 12 cm from origin was considered to have migrated 11·5 cm; this value was divided by the distance of the solvent front, 17·0 cm, yielding an R_f of 0·68.) In solvent systems I and II, ^{3}H-*S*-adenosyl-L-methionine had an R_f of 0·03 and in system III the R_f of ^{3}H-SAM was 0·18.

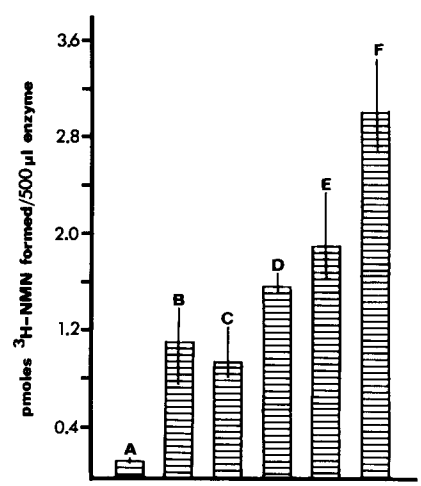

Fig. 4. Red blood cell COMT activity at 60 min of incubation in the presence of no exogenous norepinephrine (A); 0·4 mM norepinephrine (B); NE plus 1·3% (v/v) methanol (C); NE plus methanol plus 0·81 mM vernolepin (D); NE plus methanol plus 0·81 mM *n*-propanethiol (E); or NE plus methanol plus 0·81 mM bis-*n*-propanethiol adduct of vernolepin (F). Vertical bars represent the range of triplicate values obtained in three representative experiments. Statistical significance as judged by Student's *t*-test was C vs B, not significant; D vs C, $P < 0.001$; E vs C, $P < 0.02$; F vs C, $P < 0.01$.

* Scintillation mixture was prepared as follows: 1,4-bis-2-phenyloxazoyl benzene (POPOP, 0·9 g); 2,5-diphenyloxazole (PPO, 15 g); Biosolv solubilizer (BBS-3, 200 ml); and toluene to a final volume of 3 liters.

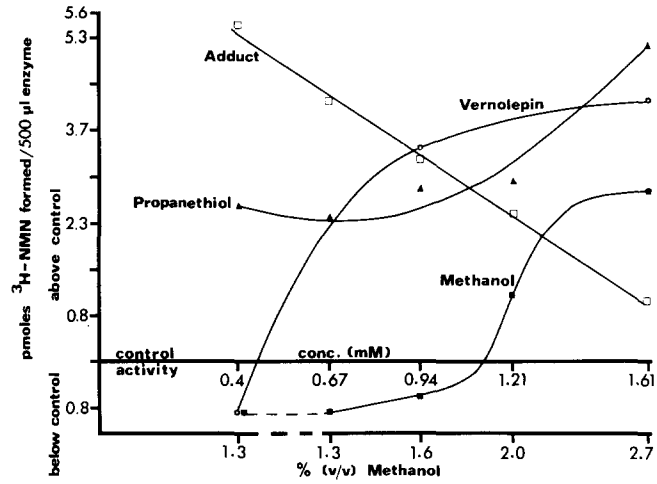

Fig. 5. Red blood cell COMT activity (at 60 min of incubation) above or below control (standard reaction mixture containing norepinephrine and ^{3}H-SAM). All contained the following components: norepinephrine, 0·4 mM; ^{3}H-SAM, 0·096 μM; $MgCl_2$, 13 mM; phosphate buffer, pH 7·9, 67 mM. In addition ■ = methanol included (concentration indicated on lowest scale); ○ = vernolepin included (concentration indicated on upper scale); ▲ = *n*-propanethiol included (concentration indicated on upper scale); □ = bis-*n*-propanethiol adduct of vernolepin included (concentration indicated on upper scale). Broken lines indicated that 1·3% (v/v) methanol was used at both 0·4 mM and 0·67 mM concentrations of vernolepin, *n*-propanethiol and adduct. Enzyme activity is indicated on the ordinate.

(bar C). Addition of vernolepin (bar D), *n*-propanethiol (bar E), or adduct (bar F) to final concentrations of 0·81 mM resulted in significant enhancements of COMT activity to levels which were, respectively, 65, 100 or 215 per cent above the methanol control.

The ability of these compounds to alter COMT activity at 60 min of incubation was next studied in terms of concentration dependence. Figure 5 shows that, at the lower methanol concentrations studied (1·3 and 1·6%, v/v), inhibition of COMT activity was observed (39 and 29 per cent respectively). At the higher methanol concentrations examined (2·0 and 2·7%, v/v), enhancement of activity was observed (46 and 122 per cent respectively). These concentrations of methanol corresponded with those required to obtain the final concentrations of vernolepin, *n*-propanethiol, or the adduct of vernolepin which were examined in Fig. 5. The concentration-dependence curve for vernolepin was similar to the curve for methanol, since at the lower concentration of vernolepin studied (0·4 mM), inhibition of activity occurred (63 per cent below control), while at the two higher concentrations of vernolepin (0·94 and 1·61 mM), stimulation of activity was observed (249 and 307 per cent above control respectively). As noted earlier, previous studies with vernolepin have shown the compound only to inhibit the activity of sulfhydryl enzymes. As can be seen in Fig. 5, both *n*-propanethiol and the adduct of vernolepin enhanced the activity of the enzyme at all concentrations examined. In the case of *n*-propanethiol, increasing concentration resulted in more enhancement of activity (maximum effect of 359 per cent above control at 1·61 mM). In contrast, the largest effect of adduct occurred at the lowest concentration examined, while the least enhancement by adduct occurred at the highest concentration examined. Thus, both vernolepin as well as its adduct are each active with respect to enhancing COMT activity. However, since the concentration-dependence patterns were different, it seems unlikely that adduct was active by virtue of conversion to the SH reactive parent compound (vernolepin). This is especially obvious at high concentrations where conversion would be favored and where it can be seen that stimulation by vernolepin is much higher than stimulation by adduct.*

We next examined the ability of the adduct to enhance COMT activity in a variety of organs at 60 min of incubation (Fig. 6). For each preparation, activity was determined in the presence of norepinephrine alone (bar A), norepinephrine and 1·3% (v/v) methanol (bar B), or norepinephrine and methanol containing adduct to a final concentration of 0·81 mM (bar C). The source of enzyme was cat erythrocyte lysate, or 78,000 *g* supernatant from the rat organ indicated in Fig. 6. In four cases, methanol caused a slight decrease in COMT activity when compared to the norepinephrine control. In one case (rat brain), methanol caused a slight increase in COMT activity when compared to the control. However, in all cases examined, the adduct caused a marked enhancement in activity when compared to the methanol control.

Figure 7 shows that the activity of a partially purified (100-fold) rat liver COMT preparation was susceptible to enhancement by these reagents also. Methanol (1·3%, v/v, bar C) produced an increase

* The possibility that the activity of adduct was due to conversion to the SH reactive parent compound, vernolepin, was ruled out by direct experiments in which ^{3}H-adduct was added to the assay mixture at time 0 followed by isolation of ^{3}H-adduct on t.l.c. (Silica gel, 15% acetone in $CHCl_3$) with no ^{3}H-vernolepin being demonstrable on t.l.c. up to 60 min of incubation.

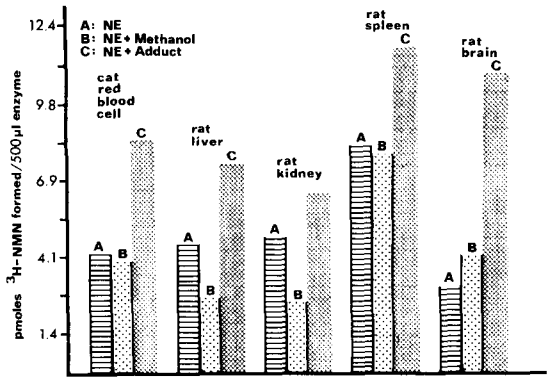

Fig. 6. COMT activity (at 60 min of incubation) in cat red blood cell lysate or 78,000 *g* supernatant of rat liver, kidney, spleen or brain. Activities were determined in the presence of 0·4 mM norepinephrine (A), norepinephrine and 1·3% (v/v) methanol (B), or norepinephrine and methanol containing 0·81 mM bis-*n*-propanethiol adduct of vernolepin (C).

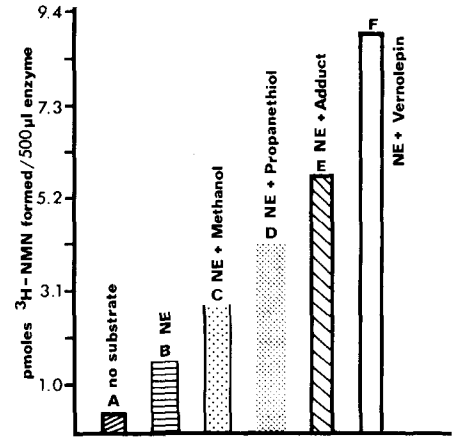

Fig. 7. COMT activity (at 60 min of incubation) in partially purified (approximately 100-fold compared to homogenate) rat liver preparation. (A) no exogenous substrate; (B) 0·4 mM norepinephrine (NE); (C) NE plus 1·3% (v/v) methanol; (D) NE plus methanol plus 0·81 mM *n*-propanethiol; (E) NE plus methanol plus 0·81 mM bis-*n*-propanethiol adduct of vernolepin; and (F) NE plus methanol plus 0·81 mM vernolepin.

in activity to a level 81 per cent above control. Addition of *n*-propanethiol (0·81 mM, bar D) resulted in an increase in activity to a level 49 per cent above the methanol control. The adduct (0·81 mM, bar E) produced an increase in activity to a level 106 per cent greater than the methanol control, while vernolepin (0·81 mM, bar F) led to an increase in activity which was 218 per cent above the methanol control.

Using conditions of linearity with time and amount of protein in the cat erythrocyte lysate COMT system, we examined whether there was an explanation for these phenomena in terms of enzyme kinetics. In the experiment in Fig. 8, we measured the apparent K_m and V_{max} using a fixed concentration of ^{3}H-*S*-adenosyl-L-methionine (^{3}H-SAM, 0·096 µM) and varying concentrations of norepinephrine (NE, 0·05 to 0·81 mM). For each concentration of NE, the activity of the enzyme was determined in the presence of NE alone, NE plus 1·3% (v/v) methanol, or NE, methanol and the adduct of vernolepin to a final concentration of 0·81 mM. The kinetic constants were estimated using a computer method with a weighted least squares regression analysis. The apparent K_m values are expressed in terms of M × 10^{-5}, while the V_{max} values are given as pmoles ^{3}H-normetanephrine formed/500 µl of enzyme/5 min of incubation. Methanol caused a slight increase in the apparent K_m for NE, while adduct increased the apparent K_m by 2- to 3-fold. Methanol had no detectable effect on the V_{max}, while

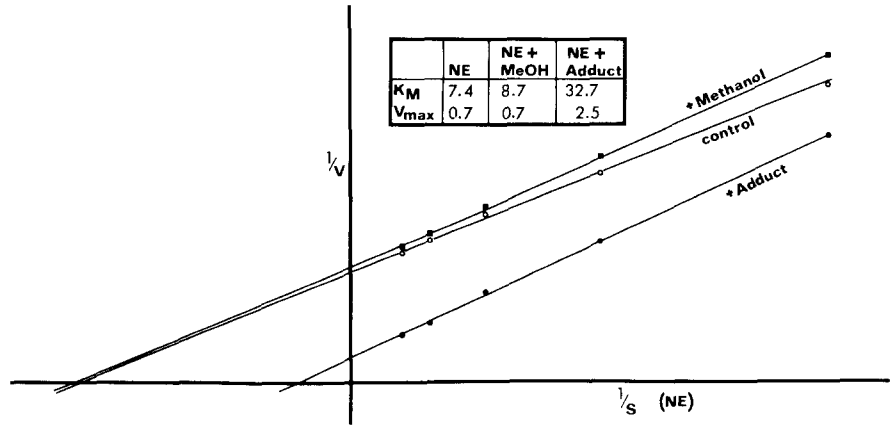

Fig. 8. Lineweaver–Burk plot derived from weighted least squares regression analysis. The experiment was performed in the presence of a fixed concentration of ^{3}H-*S*-adenosyl-L-methionine (0·096 µM) and varying concentrations of *l*-norepinephrine *d*-bitartrate (0·05–0·81 mM). The units on the abscissa are the reciprocals of norepinephrine concentrations. The estimated K_m values are expressed in terms of M × 10^{-5}, while V_{max} values are expressed as pmoles ^{3}H-normetanephrine formed/500 µl of enzyme/5 min of incubation. Control contained the following components: ^{3}H-SAM, 0·096 µM; $MgCl_2$, 13 mM; phosphate buffer, pH 7·9, 67 mM; norepinephrine, from 0·05 to 0·81 mM. In addition, ■ = methanol included (final concentration 1·3%, v/v) and ● = adduct included (final concentration 0·81 mM).

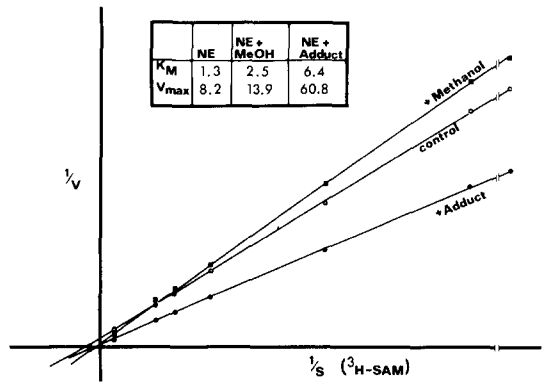

Fig. 9. Lineweaver–Burk plot derived from weighted least squares regression analysis. The experiment was performed in the presence of a fixed concentration of *l*-norepinephrine *d*-bitartrate (0·4 mM) and varying concentrations of ^{3}H-*S*-adenosyl-L-methionine (0·012 to 0·836 μM). The units on the abscissa are the reciprocals of the ^{3}H-SAM concentrations. The estimated K_m values are expressed in terms of M × 10^{-6}, while the V_{max} values are given as pmoles ^{3}H-normetanephrine formed/500 μl of enzyme/5 min of incubation. Control contained the following components: norepinephrine, 0·4 mM; $MgCl_2$, 13 mM; phosphate buffer, pH 7·9, 67 mM; ^{3}H-SAM, from 0·012 to 0·836 μM. In addition, ■ = methanol included (final concentration 1·3%, v/v) and ● = adduct included (final concentration 0·81 mM).

adduct produced a 2- to 3-fold increase in this parameter (268 per cent above control).

In the experiment in Fig. 9, we examined the effect of methanol and adduct on the kinetic constants in the presence of a fixed concentration of NE (0·4 mM) and varying concentrations of ^{3}H-SAM (0·012 to 0·836 μM). Methanol produced an approximate doubling in the apparent K_m for ^{3}H-SAM, while adduct resulted in a further 2·5-fold increase in the apparent K_m when compared to the methanol control. Methanol also caused an increase in the V_{max} to a value which was about 70 per cent greater than the control, while adduct resulted in a marked further increase in V_{max} to a level nearly 340 per cent above the methanol control.

DISCUSSION

In previous studies, only the SH reactive parent compound, vernolepin, has been found to have biological activity (tumor inhibition, inactivation of phosphofructokinase and glycogen synthase). The studies presented herein are the first report of an activity which may have biological relevance (enhancement of the activity of COMT *in vitro*) being exerted by a non-SH reactive derivative of this tumor inhibitor. Another interesting feature of this work is that vernolepin itself, which has been previously known only to inhibit the activity of sulfhydryl enzymes, has been shown to enhance the activity of COMT under some conditions. The kinetic experiments presented in the present report show that the enhancement of COMT activity exerted by the adduct of vernolepin is due to an effect on the enzyme. We believe that these kinetic changes may possibly be related to a structural change in the enzyme which may occur in the presence of this highly lipophilic reagent. The possibility that altered COMT activity was due to a chemical protective effect of adduct on either norepinephrine or ^{3}H-SAM was ruled out by measuring the recovery of NE by fluorometric assay and the recovery of ^{3}H-SAM by t.l.c. studies. Under the standard assay conditions, there was no difference in recovery of either substrate in the absence or presence of adduct. It is also noteworthy that the activity of COMT, an important enzyme in the catabolism of circulating catecholamines *in vivo*, is readily modified *in vitro* by such a variety of reagents including SH reagents (vernolepin), non-SH reactive reagents (the bis-*n*-propanethiol adduct of vernolepin) and lipophilic solvents (methanol). Other evidence exists [9] that the presence of dithiothreitol influences the kinetic properties (apparent K_m and V_{max}) of rat liver COMT *in vitro*. Because the activity of COMT *in vitro* is amenable to control by reagents of such structural diversity, we suggest that future experiments *in vivo* might be fruitful with a view to finding modifiers of COMT activity with pharmacological or therapeutic usefulness.

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