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# POSSIBLE MECHANISM OF ACTION OF SKF 64139 *IN VIVO* ON RAT ADRENAL AND BRAIN PHENYLETHANOLAMINE *N*-METHYLTRANSFERASE ACTIVITY

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**Abstract**—SKF 64139, a specific inhibitor of the epinephrine-synthesizing enzyme, phenylethanolamine *N*-methyltransferase (PNMT), has been widely used as a pharmacological tool for studying the characteristics of epinephrine-containing neurons. However, the mechanism of action of this drug on PNMT *in vivo* has not been fully elucidated. In the present study, we traced changes of PNMT activity in rat adrenal glands and medulla oblongata between 1 and 48 hr after intraperitoneal injection of SKF 64139 (50 mg/kg body wt). Within 1 hr, enzyme activity in both tissues decreased to 10% of the respective control value. However, starting at 4 hr, activity gradually recovered from the inhibition and completely returned to the respective control level by 48 hr. Removal of the inhibitor by dialysis substantially restored the adrenal enzyme activity in 1, 2 and 4 hr groups and completely returned it to control levels in 18 and 48 hr groups. A similar pattern also seemed to hold with brain extracts. The profiles of immunotitration curves, using dialyzed tissue extracts and specific antibodies to bovine adrenal PNMT, clearly indicate that, even after dialysis, a substantial amount of inactive enzyme was present in tissue extracts from 1, 2 and 4 hr groups. In contrast, by 18 hr a very small amount of inactive enzyme was present. Throughout the experimental periods there was no noticeable differences among the control and the experimental groups in the number or intensity of immunocytochemical stained neurons with PNMT antibodies of the $C_1$ area of ventrolateral medulla. Judging from the data obtained by dialysis, immunochemical titration and immunocytochemical staining, recovery of PNMT activity following its inhibition by SKF 64139 was not due to irreversible inhibition of the enzyme followed by new enzyme synthesis. Instead, reversible binding of inhibitor to PNMT and its release were responsible for recovery. PNMT from the 1, 2 and 4 hr groups resisted further *in vitro* inhibition by SKF 64139 because the residual inhibitor was probably still bound to the enzyme.

Phenylethanolamine *N*-methyltransferase (PNMT, EC 2.1.1.28, *S*-adenosyl-L-methionine:phenylethanolamine *N*-methyltransferase), the enzyme catalyzing the conversion of norepinephrine to epinephrine, is present in the adrenal medulla, retina and discrete regions in the brain [1–11].

PNMT has been purified from the adrenal glands and well characterized biochemically, immunochemically and molecular biologically [1, 12–18]. Recently, the complete amino acid sequence of bovine adrenal PNMT was deduced from the nucleotide sequence of bovine adrenal PNMT complementary deoxyribonucleic acid [19].

SKF 64139 (7,8-dichloro-1,2,3,4-tetrahydroisoquinoline), a potent and reversible inhibitor of PNMT both *in vitro* and *in vivo* [20], has been widely used as a pharmacological tool for studying the functions of epinephrine-containing neurons. The drug is a competitive inhibitor with respect to norepinephrine and uncompetitive to *S*-adenosylmethionine [20]. Its administration also appears to lower epinephrine levels as well as PNMT activity in the CNS [21, 22]. However, the mechanism of action of this inhibitor on PNMT *in vivo* has not been fully understood.

To elucidate the mechanism of action of the drug on PNMT inhibition *in vivo*, we performed the present study to determine: (1) the time course of changes in PNMT activity in rat adrenal gland and medulla oblongata between 1 and 48 hr after intraperitoneal injection of SKF 64139; (2) changes of enzyme activity in dialyzed tissue extracts from the drug-treated animals; (3) the presence of inactive enzyme in dialyzed tissue extracts by immunotitration, using a specific PNMT antiserum; (4) whether the intensity and number of immunocytochemically stained $C_1$ neurons in control and SKF 64139 treated animals are equivalent; and finally (5) whether after dialysis the enzyme exposed to the inhibitor *in vivo* is inhibited further by *in vitro* treatment. From these experiments we will show that the recovery of PNMT activity following *in vivo* inhibition by SKF 64139 treatment was due to a gradual release of enzyme bound inhibitor and not to newly synthesized enzyme.

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## MATERIALS AND METHODS

Male Sprague–Dawley rats (Hilltop Lab. Animals, Scottdale, PA) weighing 200–240 g were used throughout this study. The animals were injected intraperitoneally with either 0.9% (w/v) NaCl or SKF 64139 in 0.9% (w/v) NaCl (50 mg/kg body wt) and were killed by decapitation after various time periods.

Adrenal glands and medulla oblongata were quickly dissected as described previously [23]. Medulla oblongata or adrenal glands were homogenized in either 300 μl or 2 ml, respectively, of 10 mM potassium phosphate buffer, pH 7.0, containing 0.2% (v/v) Triton X-100. Homogenates were centrifuged at 9750 g for 10 min. The resulting supernatant fractions were used for assay of PNMT activity, protein determination [24], and immunochemical titration.

The PNMT assay was carried out as described previously [17, 25]. Tissue extracts were dialyzed, if necessary, in 10 mM potassium phosphate buffer, pH 7.0, for 2 hr with one change of the buffer. Preparation of antiserum against bovine adrenal PNMT has been described in detail elsewhere [14]. The immunochemical titration was carried out by adding increasing amounts of antiserum to appropriately diluted 50-μl aliquots of dialyzed tissue extract. The rest of the procedure is as described previously [14, 25]. Immunocytochemical staining of PNMT neurons was performed by the peroxidase–antiperoxidase technique as described in detail elsewhere [6]. For the *in vitro* inhibition study, PNMT activity was assayed in the presence of $1 \times 10^{-7}$ or $1 \times 10^{-5}$ M SKF 64139.

Statistical analysis was carried out by using Student's *t*-test.

## RESULTS

Following a single i.p. injection of SKF 64139, changes of PNMT activity in the adrenal gland and medulla oblongata were monitored between 1 and 48 hr. As shown in Table 1, PNMT activity in both tissues was decreased to about 10% of the respective control levels within 1 hr of administration. The degree of PNMT inhibition remained constant during the first 2 hr. Starting from 4 hr after treatment, enzyme activity markedly recovered from inhibition in both tissues. By 18 hr after treatment, adrenal enzyme activity was restored to almost 90% of control level, while brain enzyme activity was restored to only 60% of control. Despite this difference in the rate of recovery, PNMT activity in both tissues was fully restored to the respective control level within 48 hr after treatment.

To determine whether or not this inhibition was reversible, adrenal extracts from animals treated with 0.9% (w/v) NaCl or SKF 64139 were subjected to dialysis against 10 mM potassium phosphate buffer, pH 7.0, for 2 hr prior to determination of PNMT activity (Table 2). In 1, 2 and 4 hr groups, dialysis resulted in a substantial recovery of PNMT activity, although 100% recovery was not obtained. In 18 and 48 hr groups, the enzyme activity returned to the control levels following dialysis.

Immunotitration curves of PNMT activity (100% equals 23 pmol/15 min) from dialyzed adrenal extracts of control and SKF 64139 treated animals (1, 2, 4, 18 and 48 hr after treatment) are illustrated in Fig. 1A. The percentage of control activity was plotted against the amount of antiserum used. The slope of the immunotitration curve is the steepest with the control and 48 hr groups and the least steep with the 1 hr group. Control, 48, 18, 4, 2 and 1 hr

Table 2. Partial recovery by dialysis of PNMT activity from rat adrenal following i.p. administration of SKF 64139

| Hour after injection | PNMT activity (nmol/mg protein/15 min) | % of Control activity |
|---|---|---|
| Control | 1.107 ± 0.0930 (9) | 100 |
| 1 | 0.555 ± 0.0250*(9) | 50.0 |
| 2 | 0.659 ± 0.0458*(9) | 59.5 |
| 4 | 0.877 ± 0.0378†(9) | 79.2 |
| 18 | 1.128 ± 0.0297 (8) | 102 |
| 48 | 1.181 ± 0.0139 (8) | 107 |

Animals were treated with SKF 64139 (50 mg/kg, i.p.). Dialysis was carried out in 10 mM phosphate buffer, pH 7.0, for 2 hr with one change of the buffer. Results are the mean ± SEM of the number shown in parentheses.

*, † Significantly different from control at: * $P < 0.001$, † $P < 0.05$.

Table 1. Time course for inhibition of PNMT activity in rat adrenal gland and medulla oblongata following i.p. administration of SKF 64139

| | Adrenal gland | | Medulla oblongata | |
|---|---|---|---|---|
| Hour after injection | PNMT activity (nmol/mg protein/15 min) | % of Control activity | PNMT activity (pmol/mg protein/15 min) | % of Control activity |
| Control | 1.075 ± 0.0302 (59) | 100 | 15.6 ± 0.443 (39) | 100 |
| 1 | 0.136 ± 0.0135*(19) | 12.7 | 1.85 ± 0.309*(10) | 11.9 |
| 2 | 0.169 ± 0.0079*(19) | 15.7 | 2.50 ± 0.291*(10) | 16.0 |
| 4 | 0.426 ± 0.0288*(19) | 39.6 | 4.55 ± 0.348*(10) | 29.2 |
| 18 | 0.943 ± 0.0383†(19) | 87.7 | 8.91 ± 0.626*(10) | 57.1 |
| 48 | 1.026 ± 0.0260 (19) | 95.4 | 16.6 ± 1.056 (8) | 106 |

Animals were treated with SKF 64139 (50 mg/kg, i.p.). Results are the mean ± SEM of the number shown in parentheses.

*, † Significantly different from control at: * $P < 0.001$, and † $P < 0.05$.

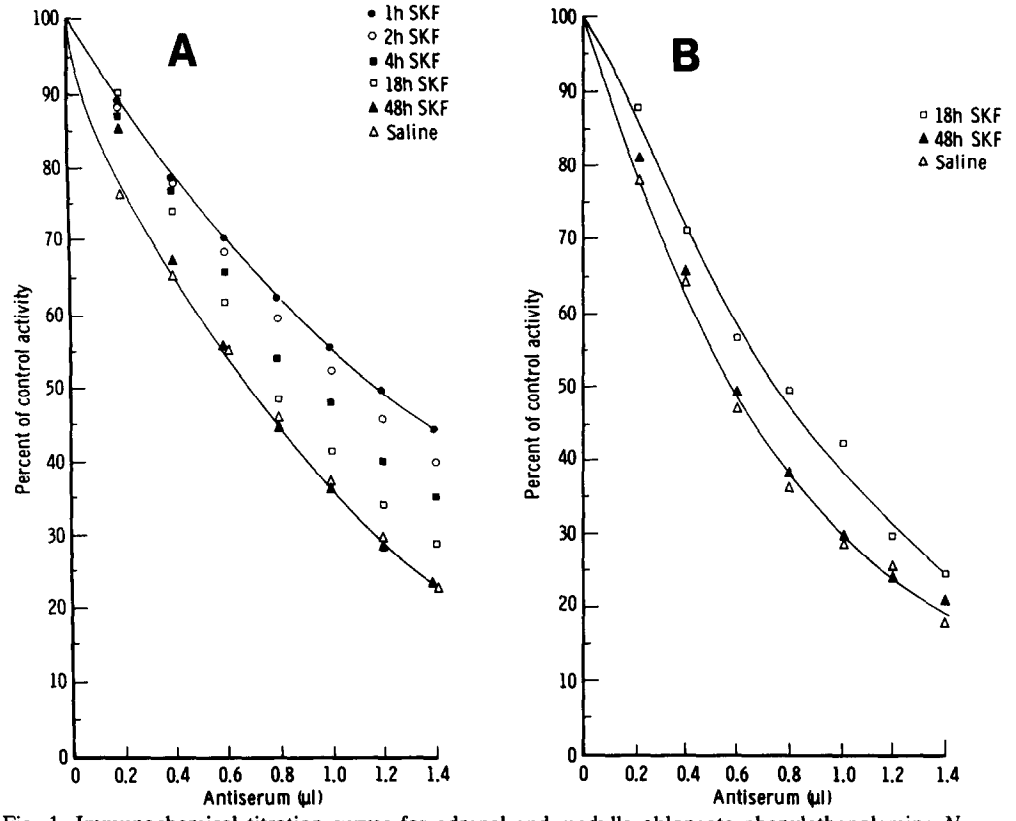


Fig. 1. Immunochemical titration curves for adrenal and medulla oblongata phenylethanolamine *N*-methyltransferase from 0.9% (w/v) NaCl or SKF 64139 treated animals. Dialyzed adrenal or medulla oblongata extracts from animals injected with either saline or the inhibitor were used for immunotitration. The presence of immunotitration curves with differing slopes indicates that an enzymatically inactive but immunoreactive form of the enzyme is present only in tissue extracts from inhibitor-treated animals even after dialysis. (A) Adrenal extracts (100% equals 23 pmol/15 min); and (B) medulla oblongata extracts (100% equals 13 pmol/15 min).

groups are in a progressively decreasing order of steepness in slope. The change in slopes of the 18, 4, 2 and 1 hr groups indicates that more inactive PNMT was present in those groups than in the control and 48 hr groups. A similar immunotitration curve profile was also seen with PNMT (100% equals 13 pmol/15 min) from dialyzed medulla oblongata extracts, as illustrated in Fig. 1B. Curves for the 1, 2 and 4 hr groups were not included, because of the difficulty of performing immunotitration in tissue with such low enzyme activity.

To determine if the drug altered the amount of PNMT immunoreactivity, serial sections of medulla oblongata from animals treated with saline or SKF 64139 (2 and 18 hr prior to sacrifice) were stained with specific antibodies to bovine adrenal PNMT by the peroxidase–antiperoxidase technique. There was no difference observed in either intensity or number of immunocytochemically stained neurons (perikarya or processes) in the $C_1$ area of the ventrolateral medulla among the control and SKF 64139 treated groups, as illustrated in Fig. 2. Furthermore, no change in intensity or number of stained cells was seen in serial sections of the $C_2$ area (data not shown).

The possibility that the drug was bound to PNMT, thus forming an inactive enzyme, was studied in adrenal extracts from animals pretreated with the inhibitor and assayed for PNMT activity after dialysis in the absence or presence of $1 \times 10^{-7}$ M SKF 64139 *in vitro*. As illustrated in Table 3, the addition of the drug *in vitro* to nondialyzed extracts resulted in much

Table 3. Further inhibition by SKF 64139 *in vitro* of adrenal PNMT activity in inhibitor-treated rats

| Hour after injection | PNMT activity (nmol/mg protein/15 min) | | | |
|---|---|---|---|---|
| | Nondialyzed | | Dialyzed | |
| | (−) | (+) | (−) | (+) |
| 1 | 0.0773 | 0.0642 | 0.470 | 0.222 |
| 2 | 0.125 | 0.103 | 0.541 | 0.242 |
| 4 | 0.265 | 0.168 | 0.696 | 0.269 |
| 18 | 0.707 | 0.277 | 0.959 | 0.304 |
| 48 | 0.828 | 0.273 | 0.883 | 0.298 |
| Control | 0.821 | 0.261 | 0.880 | 0.263 |

PNMT activity was determined in duplicates. The animals were administered SKF 64139 (50 mg/kg, i.p.). Dialysis was carried out in 10 mM potassium phosphate buffer, pH 7.0, for 2 hr with one change of the buffer. For *in vitro* inhibition $1 \times 10^{-7}$ M SKF 64139 was added to the PNMT assay mixture. Key: (−) symbol indicates absence of inhibitor; (+) symbol indicates presence of inhibitor.

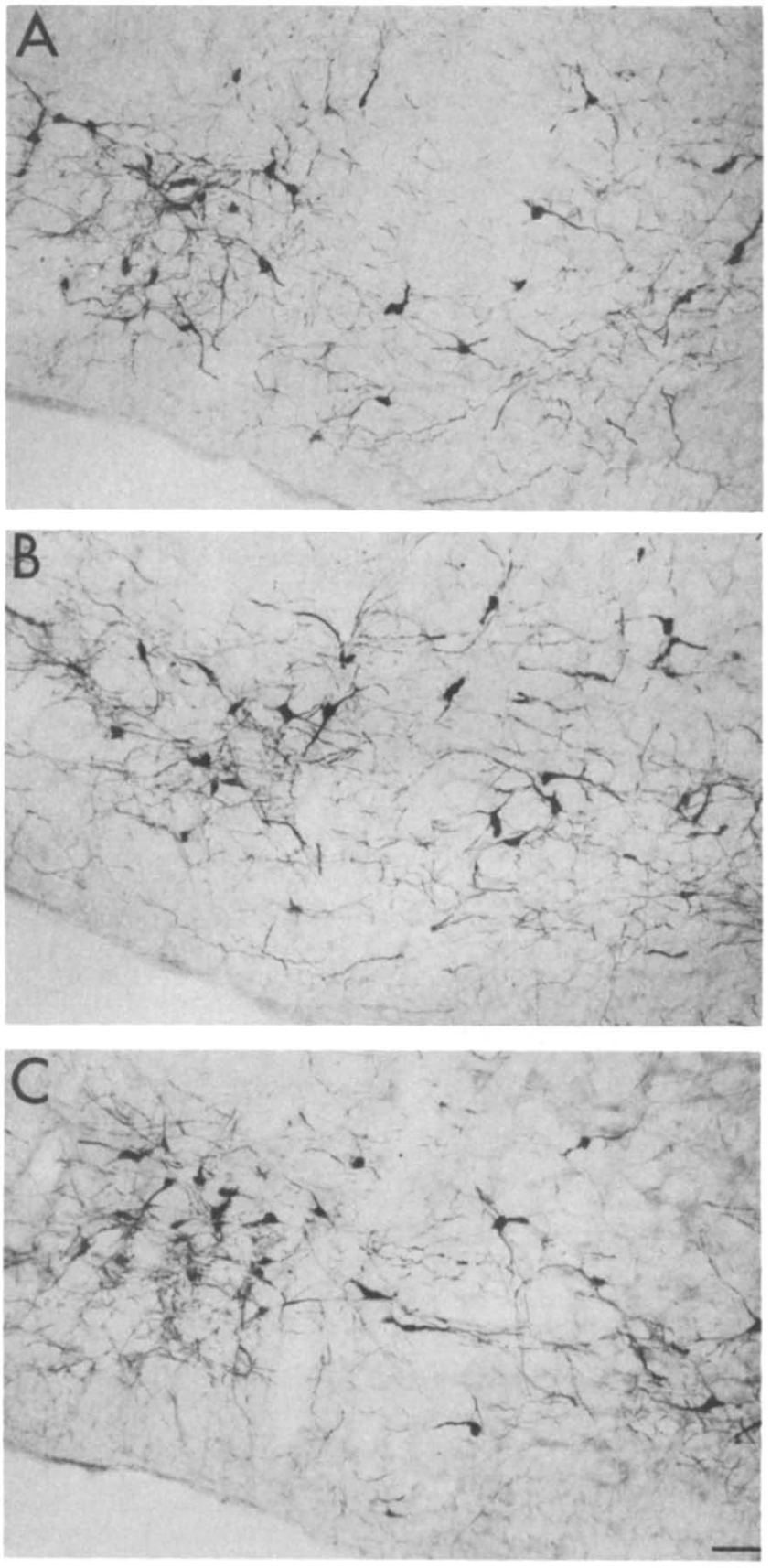


Fig. 2. Immunocytochemically stained phenylethanolamine *N*-methyltransferase-containing neurons in the $C_1$ area of saline or SKF 64139 treated animals. Sections from animals treated with saline or the inhibitor (2 and 18 hr prior to sacrifice) were stained with specific antibodies to PNMT by the peroxidase–antiperoxidase technique. In all three groups, the intensity and number of immunocytochemically stained neurons were equivalent in the $C_1$ area of the ventrolateral medulla. (A) Controls; (B) 2 hr; and (C) 18 hr. Bar = 50 $\mu$m.

larger inhibition of PNMT activity in extracts of the 18, 48 hr and control groups than of the 1, 2 and 4 hr groups. Similar patterns of *in vitro* inhibition were also observed with dialyzed extracts. However, *in vitro* inhibition of PNMT activity by SKF 64139 was noticeably greater in dialyzed tissue extracts than in nondialyzed samples. On the other hand, PNMT activity of nondialyzed or dialyzed adrenal extracts was inhibited completely *in vitro* in the presence of $1 \times 10^{-5}$ M SKF 64139.

## DISCUSSION

The results obtained in the present study clearly demonstrate that SKF 64139 administration inhibited not only peripheral but also central PNMT activity *in vivo* almost completely within 1 hr after its administration, and that enzyme levels were fully restored within 48 hr. Although adrenal and brain PNMT activities were fully returned to control levels within 48 hr after administration of the inhibitor, the initial recovery rate of the adrenal enzyme activity through 18 hr was faster than that of the brain enzyme.

In support of the above observations is the demonstration that, about 1 hr after administration of SKF 64139, the drug markedly inhibits the conversion of a tracer dose of [$^3$H]-norepinephrine to [$^3$H]-epinephrine in the rate adrenal gland [20]. The studies indirectly indicate *in vivo* inhibition of PNMT activity by the drug. Our time course for inhibition of brain PNMT activity generally agrees with the findings of Pendleton *et al.* [21] and Sauter *et al.* [22] despite differences in the experimental conditions used.

The question we asked in this study was: is the *in vivo* inhibition of PNMT activity by SKF 64139 and its full recovery due to either irreversible enzyme inhibition followed by the appearance of newly synthesized enzyme, or inhibitor binding to PNMT and then a gradual inhibitor release from the enzyme? To answer these questions, we chose a combined biochemical, immunochemical and immunocytochemical approach.

As shown in Table 2, removal of the inhibitor by dialysis alone led to a substantial restoration of adrenal enzyme activity in all of the experimental periods. In the 1, 2 and 4 hr groups, full restoration of enzyme activity to the control level was not obtained, probably due to the difficulty in complete removal of the tightly bound inhibitor. In the 18 and 48 hr groups, on the other hand, complete recovery of activity occurred.

Matched enzyme activity of control and experimental groups was used for immunotitration. Thus, the following can be predicted: (1) the immunotitration curves of both control and experimental groups should overlap, if there is no inactive enzyme present in the inhibitor-treated groups; (2) the presence of both catalytically active and inactive forms of the enzyme is indicated when the immunotitration curve has a shallow slope, compared to the control curve; and (3) the presence of lower amounts of catalytically active and inactive enzyme is indicated by a steeper slope, when compared to the control.

Even after partial removal of the inhibitor by dialysis, adrenal extracts from the 1, 2 and 4 hr groups contained a substantial amount of inactive enzyme as indicated by the shallower slopes of the immunotitration curves. Adrenal and medulla oblongata extracts from the 18 hr group still contained a very small amount of inactive enzyme, but by 48 hr no inactive enzyme was found.

The fact that PNMT activity gradually returned to control levels following treatment and that the total amount of immunoreactive enzyme protein (the sum of catalytically active and inactive enzyme protein) appeared to remain constant throughout the experimental periods suggest that the inhibitor is initially bound to the enzyme and then gradually released *in vivo*. Even if *de novo* synthesis of PNMT occurred within the 48 hr period after inhibitor administration, its amount was minimal. This conclusion is based on our observation that the total activity of 18 and 48 hr groups after removal of inhibitor by dialysis never exceeded the control level. Furthermore, the number or intensity of $C_1$ and $C_2$ neurons stained immunocytochemically with PNMT antibodies did not differ among control, 2, 4 (not shown) and 18 hr groups. This observation again suggests that during the experimental periods there was no noticeable change in the total amount of immunoreactive enzyme protein.

In this study it was found that, at 1–4 hr after *in vivo* treatment, the inhibitor appeared to be maximally and tightly bound to PNMT since further complete inhibition could not be produced *in vitro* even after dialysis except when an excessive amount of the inhibitor was used. Therefore, our results demonstrate that the recovery of PNMT activity after SKF 64139 treatment *in vivo* is attributable to gradual reversible removal of the inhibitor from the enzyme protein.

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