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# DOG LIVER *N*-METHYLTRANSFERASE

## A DRUG-METABOLIZING ENZYME

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**Abstract**—A non-specific *N*-methyltransferase was demonstrated in dog liver. This enzyme is different from other *N*-methylating systems, especially in terms of substrate and species specificity. The enzyme catalyzes the methylation of a variety of endogenous and exogenous amines; of the compounds studied, SK&F 64139 (7,8-dichloro-1,2,3,4-tetrahydroisoquinoline) was found to be the best substrate. The enzyme utilized *S*-adenosylmethionine but not 5-methyltetrahydrofolate as a methyl donor, and it had a pH optimum at 8.0. Study of SK&F 64139 with the partially purified enzyme indicated that this dog liver *N*-methyltransferase had very low $K_m$ and high $V_{max}$ values for SK&F 64139. Methylation of SK&F 64139 was not observed with the monkey or rat liver enzyme preparation. This finding is in accordance with the fact that SK&F 64139 is methylated extensively in the dog, but not in other species. The ability of this enzyme to methylate a number of arylalkylamines suggests its possible importance in drug biotransformation.

Since a non-specific *N*-methyltransferase in rabbit lung was described by Axelrod [1], a number of other *O*- and *N*-methylation enzymes have been reported [2], including histamine *N*-methyltransferase, phenylethanolamine *N*-methyltransferase (PNMT), indoleamine *N*-methyltransferase and catechol-*O*-methyltransferase. These enzymes appear to be of greater importance in the biotransformation of endogenous compounds than in the metabolism of drugs. Recently, Pendleton *et al.* [3] reported species differences in the enzyme systems for the *N*-methylation of phenethylamine-type compounds in pulmonary tissues. The similarities and differences between these systems and PNMT were described. However, the role of these pulmonary enzymes is still unknown. SK&F 64139, a potent inhibitor, but a very poor substrate for PNMT [4], was found to be methylated extensively in dog after intravenous or oral administration [5]. The compound was also reported to be an inhibitor, but not a substrate, for the *N*-methyltransferase from dog lung [3]. These findings led us to investigate the enzyme responsible for the methylation of SK&F 64139. The present paper describes the results of our investigation and the partial purification and characterization of a newly discovered *N*-methyltransferase in dog liver. The enzyme also catalyzes the methylation of a number of foreign amines and exhibits different substrate specificity from other methyltransferases. It is concluded that this enzyme might be important in the metabolism of exogenous compounds.

### MATERIALS AND METHODS

Carbon-14 labeled SK&F 64139 (sp. act. 6.4 mCi/mmole) was synthesized by Dr. W. Mendelson in the Smith Kline & French Laboratories [1]. *S*-Adenosyl-L-methionine hydroiodide (SAM) was obtained from the Sigma Chemical Co., St. Louis, MO, and [$^{14}CH_3$]SAM (sp. act. 58 mCi/mmole) was purchased from Amersham, Arlington Heights, IL. 3-*O*-Methylepinine was obtained from the Smith Kline & French Laboratories. Compounds without SK&F numbers were all from the Sigma Chemical Co.; enzyme grade ammonium sulfate was from the Mann Research Laboratory, Orangeburg, NY.

*Animals.* Mongrel dogs, weighing approximately 20 kg, were administered pentobarbital (30 mg/kg) intravenously before being killed. The liver was removed immediately, thoroughly rinsed with ice-cold saline, and kept in ice-cold saline until processing.

Male Cynomolgus monkeys (*Macaca fascicularis*) were immobilized with Kataset (15 mg/kg, intramuscularly) and then anesthetized with 30 mg/kg pentobarbital, given intramuscularly before sacrifice. Male CD Charles River rats, weighing about 300 g, and CD-1 mice (Charles River, 30 g) were killed by decapitation.

*Enzyme preparation.* Fresh liver or fresh pooled liver was rinsed thoroughly with cold saline and then homogenized in 1.15% potassium chloride (2 ml/g tissue) in a Potter-type homogenizer with a motor-driven teflon pestle. The homogenate was centrifuged for 20 min at 9000 *g* in a Sorvall RC2-B centrifuge. The supernatant fraction (9000 *g*-S) was recentrifuged at 100,000 *g* for 60 min using a refrigerated Beckman Ultracentrifuge to yield cytosol (supernatant) and microsomes (pellet) which were resuspended in 1.15% KCl (1 ml/g tissue). The enzyme was partially purified by ammonium sulfate fractionation. Ammonium sulfate (7.2 g) was dissolved in the cytosol preparation (30 ml) by gentle stirring. After standing in ice for 30 min, the mixture was centrifuged at 4000 *g* for 15 min. The supernatant

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fraction was removed, and additional ammonium sulfate (4 g) was added. The precipitate was collected by centrifugation (4000 *g*, 15 min) and then redissolved in 0.05 M sodium phosphate (pH 7.4) to a final volume of 15 ml. This 40–60% ammonium sulfate fraction was used in most assays unless stated otherwise. All preparations were stored in small aliquots at −70° until use, and the enzyme (crude or partially purified) was found to be stable for at least 2 months under these conditions.

*Enzyme assay*. The standard incubation mixture (250 μl) contained 15 μmoles of sodium phosphate (pH 8.0), 50 nmoles of substrate, 50 or 250 nmoles of [$^{14}$C]SAM (200,000 dpm), and 50 μl of enzyme (equivalent to 50 mg of liver tissue). Incubation was carried out at 37° for 30 min or 1 hr. Reaction was stopped by the addition of saturated sodium borate (pH 10), and the product was extracted with ethyl acetate (2 × 2 ml). The organic layers (1.5 ml from each extraction) were combined and evaporated to dryness. The residue was dissolved in 10 ml of Bray's solution and counted in a Packard Tri-Carb scintillation spectrophotometer. Counting efficiency was about 75%. The recovery of *N*-methyl-SK&F 64139 in this extraction procedure was about 87–90%. When [$^{14}$C]SK&F 64139 (6.4 mCi/mmole) was used as substrate, the reaction was stopped by the addition of 2 vol. of cold absolute ethanol to the mixture. The precipitated protein was separated by centrifugation, and the supernatant fraction was analyzed by high pressure liquid chromatography (HPLC). The HPLC system consisted of a Whatman Partisil 10 ODS-3 column (4.6 × 250 mm) and a Radiomatic radioactive flow detector (Radiomatic Flo-One). The solvent system was 0.05 M ammonium acetate (pH 4.5)–methanol (30:70, v/v) with a flow rate of 1 ml/min. The methylated product was identified by comparing the retention volume with that for the authentic *N*-methyl-SK&F 64139. The kinetic parameters, $K_m$ and $V_{max}$, of the Michaelis–Menten equation were determined by least squares analysis using a Hewlett–Packard computer program.

Table 1. Subcellular distribution of dog liver *N*-methylating enzyme activity using [$^{14}$C]SK&F 64139 (0.1 mM) as substrate and SAM (0.5 mM) as a methyl donor*

| Fractions | Activity [nmoles·(g tissue)$^{-1}$·hr$^{-1}$] |
|---|---|
| 9000 *g*-S | 189 |
| Cytosol | 204 |
| Microsomes | 0 |
| 40–60% Ammonium sulfate fraction from cytosol | 141 |

* Reaction was carried out at 37° for 1 hr and was terminated by the addition of 2 vol. of ethanol. The degree of methylation was measured by HPLC with a u.v. and radioactivity monitor. The data are from a representative dog liver tissue and are the average of two experiments.

## RESULTS

*Subcellular distribution and species specificity*. For all the dog livers studied, the non-specific *N*-methyltransferase was found in the cytosol fraction. In Table 1 are data from one representative dog liver tissue, showing that no enzyme activity was present in the microsomal fraction. The results from ammonium sulfate fractionation also indicated that most of the enzyme activity was associated with the protein fraction precipitated by 40–60% ammonium sulfate. It was also observed that the enzyme level was different in individual dogs (Table 2). Factors contributing to this difference have not been investigated. No activity could be detected in the monkey or rat liver, using either [$^{14}$C]SAM or [$^{14}$C]SK&F 64139 as a marker in the assay. Comparatively low but significant activity was detected in the mouse liver cytosol (sp. act. 25 ± 5 nmoles per g tissue per hr).

*Enzyme properties*. *S*-Adenosylmethionine but not 5-methyltetrahydrofolate acted as a methyl donor for the enzyme, and no requirement of $Mg^{2+}$ for activity could be noted (Table 3). A pH optimum

Table 2. *N*-Methyltransferase activity in dog liver*

| Breed | Sex | Weight (kg) | Activity [nmoles·(g tissue)$^{-1}$·hr$^{-1}$] |
|---|---|---|---|
| Mongrel | M | 20 | 396 |
| Mongrel | M | 21 | 182† |
| Mongrel | M | ‡ | 28 |
| Mongrel | F | 20.5 | 374† |
| Mongrel | F | 23 | 434 |
| German shepherd | M | 29.5 | 747 |

* Assays were performed within 24 hr after the liver was processed. The reaction mixture contained 0.1 mM SK&F 64139 and 0.5 mM [$^{14}$C]SAM in 0.06 M sodium phosphate (pH 8.0). The methylation reaction was carried out at 37° for 1 hr and then terminated by the addition of 0.5 M sodium borate (pH 10). The $^{14}$C-methylated product was extracted with ethyl acetate twice. The combined extracts were evaporated to dryness and counted in Bray's solution. Data are the average of duplicate determinations.

† Average of two experiments.

‡ Not determined; the dog was about 3 months old.

Table 3. Effect of 5-methyltetrahydrofolate (MTHF) and $Mg^{2+}$ on the methylation of [$^{14}$C]SK&F 64139 with dog liver *N*-methyltransferase*

| Additions | % of methylation | Enzyme activity (% control) |
|---|---|---|
| SAM, 0.5 mM (control) | 55.0 | 100 |
| MTHF, 0.5 mM, no SAM | 0 | |
| MTHF, 0.1 mM, no SAM | 0 | |
| $MgCl_2$, 10 mM, SAM 0.5 mM | 50.2 | 91.2 |
| $MgCl_2$, 5 mM, SAM 0.5 mM | 49.5 | 90.0 |

* Reaction mixture containing 0.1 mM [$^{14}$C]SK&F 64139 and appropriate additions in 0.06 M phosphate buffer (pH 8.0) was incubated at 37° for 1 hr. The percentage of methylation was measured as described in Table 1. Data are the average of two experiments.

of 8.0 was determined in phosphate buffer (Fig. 1), and the reaction was linear with time up to 1 hr under the standard assay conditions (Fig. 2).

*Substrate specificity*. The enzyme was found to catalyze the *N*-methylation of several naturally occurring compounds (Table 4). Epinephrine, dopamine and deoxyepinephrine (epinine) were methylated quite rapidly. Norepinephrine, phenylethanolamine and phenethylamine were methylated at a much slower rate, and histamine, tryptamine and *N*-methyltryptamine were not substrates at all.

In addition to SK&F 64139, other tetrahydroisoquinoline derivatives and PNMT inhibitors [6] were also found to be good substrates for the enzyme (Table 5). However, it is interesting to note that SK&F 29661, another very potent PNMT inhibitor [7], was methylated much more slowly than SK&F 64139. When benzylamine-type compounds were tested as substrates, differences in substrate efficiency (10×) were observed between the position isomers (Table 5).

*N-methylation of SK&F* 64139. Among all the compounds tested, SK&F 64139 was found to be the best substrate for the dog liver *N*-methylation enzyme, as indicated by the low $K_m$ and the high methylation rate. The $K_m$ value for SK&F 64139 with the partially purified enzyme was about $3.3 \pm 0.1 \times 10^5$ M, using either [$^{14}$C]SK&F 64139 or [$^{14}$C]SAM in the assay (Figs. 3 and 4). The $K_m$ value for SK&F 64139 was about the same at different concentrations of SAM, suggesting that the binding of the compound to the enzyme was not affected by

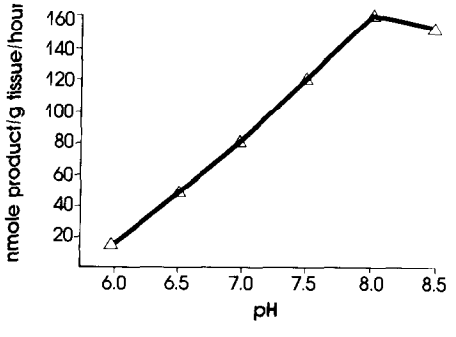



Fig. 1. pH optimum of *N*-methylation of [$^{14}$C]SK&F 64139 (0.1 mM) with partially purified dog liver *N*-methyltransferase in phosphate buffer. The experimental details were as described in Table 1.

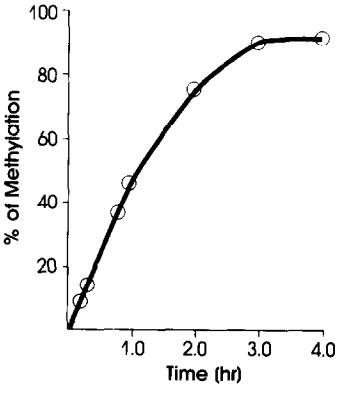



Fig. 2. Rate of *N*-methylation of [$^{14}$C]SK&F 64139 with partially purified dog liver *N*-methyltransferase. The incubation mixture contained 0.1 mM substrate and 0.5 mM SAM in pH 8.0 phosphate buffer. The experimental details were as described in Table 1.

Table 4. Substrate specificity of partially purified dog liver *N*-methyltransferase*

| Substrate | Activity as % of SK&F 64139 |
|---|---|
| SK&F 64139 | 100 |
| Phenylethanolamine | 1.7 |
| β-Phenethylamine | 2.2 |
| Norepinephrine | 5.3 |
| Epinephrine | 15.2 |
| Dopamine† | 14.1 |
| Deoxyepinephrine† (epinine) | 30.1 |
| Histamine | 0.5 |
| Tryptamine | 0 |
| *N*-Methyltryptamine | 0 |

* Reaction mixture contained 0.1 mM substrate and 0.5 mM SAM in 0.06 M sodium phosphate (pH 8.0). Methylation reaction was carried out at 37° for 1 hr and then terminated by the addition of 0.5 M sodium borate (pH 10). The $^{14}$C-methylated product was extracted with ethyl acetate twice. The combined extracts were evaporated to dryness and counted in Bray's solution. The activity with SK&F 64139 was 219 ± 2.0 nmoles per g tissue per hr. Data are means of three experiments.

† The ethyl acetate extracted $^{14}$C-methylated product was analyzed by HPLC on a Whatman Partisil ODS-3 column (4.6 × 250 mm), with a solvent system of 2.5% (v/v) methanol in 0.05 N ammonium acetate (pH 4.5). Using *O*-methyldopamines and *O*-methylepinine as references, no *O*-methylation was detected under the conditions studied.

Table 5. Substrate efficiency of SK&F 64139 and related compounds with dog liver *N*-methyltransferase*

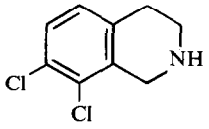
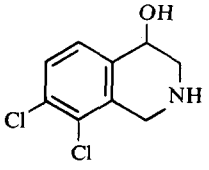
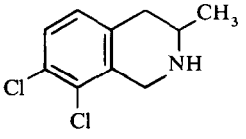
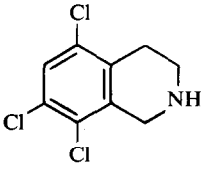
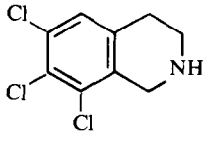
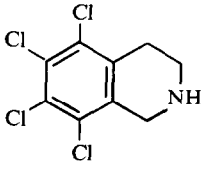
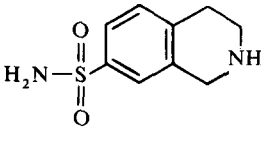
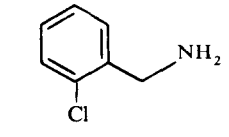
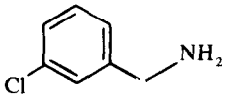
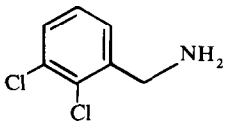
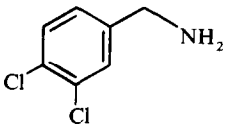
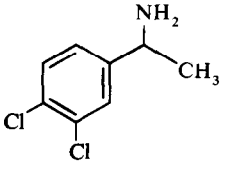
| Substrate (0.1 mM) | SK&F No. | Activity as % of SK&F 64139 |
|---|---|---|
|  | 64139 | [117 ± 10 nmoles·g( tissue)$^{-1}$·hr$^{-1}$] |
|  | 74687 | 94.1 |
|  | 84050 | 61.4 |
|  | 82431 | 21.4 |
|  | 82519 | 27.9 |
|  | 86556 | 12.5 |
|  | 29661 | 0.6 |
|  | 34240 | 41.7 |
|  | 57369 | 4.3 |

Table 5–*continued*

| Substrate (0.1 mM) | SK&F No. | Activity as % of SK&F 64139 |
|---|---|---|
|  | 63602 | 23.6 |
|  | 73115 | 2.1 |
|  | 77581 | 11.8 |

* The experimental details were as described in Table 3. Data are means of three experiments.

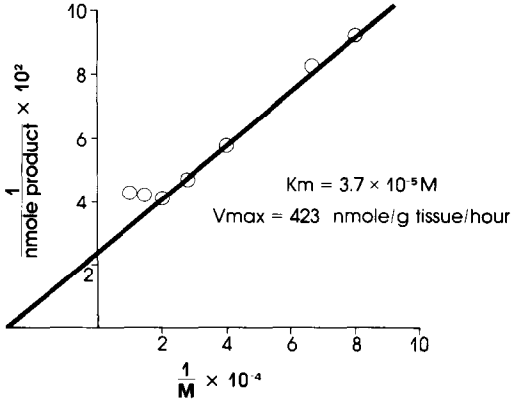



Fig. 3. Lineweaver–Burk plot of the *N*-methylation of [$^{14}$C]SK&F 64139 with partially purified dog liver *N*-methyltransferase. The concentration of SAM was 0.5 mM, and the incubation was about 15–30 min. Measurement of product formation was as described in Table 1.

SAM, and that a ping–pong mechanism was not involved.

## DISCUSSION

The data presented in this paper demonstrate that the dog liver contained an enzyme which catalyzed the methylation of both exogenous and endogenous amines. This enzyme was different from other *N*-methylating systems reported in the literature, especially with respect to substrate specificity. The dog lung contains an enzyme that has a substrate specificity similar to that of PNMT for which phenylethanolamine is a substrate but for which β-phenethylamine and SK&F 64139 are not [3]. The enzyme in rabbit lung is non-specific. Both phenylethanolamine and β-phenethylamine are effective as substrates at equivalent concentrations, and the enzyme is not inhibited by SK&F 64139 but catalyzes the methylation of the compound instead. However, this rabbit lung enzyme has been reported to have very high affinity for the indoleamines, such as tryptamine and *N*-methyltryptamine [8], whereas these two compounds were essentially inactive as a substrate for the dog liver *N*-methyltransferase. On the other hand, there were some similarities among these three *N*-methylating enzymes: (i) they were present in the tissue soluble fraction (cytosol), (ii) their pH optima were around 8.0, and (iii) they required SAM, but not $Mg^{2+}$, for activity.

The results presented in Table 5 suggest that this dog liver enzyme could play a significant role in the methylation of exogenous amines. Of the compounds studied, SK&F 64139 was found to be the best substrate; other 7,8-dichloro-substituted compounds were also effective substrates for the enzyme. The tri-chloro and tetra-chloroisoquinolines, however, were methylated more slowly than SK&F 64139. This reduction in methylation rate might be due to the steric effect of the additional chlorine substituents. SK&F 29661, which has a sulfonamide group at the 7-position, was a very poor substrate also. Since the sulfonamide group is bulky and hydrophilic, it could hinder the binding of SK&F 29661 to the enzyme. These data suggest the possibility of a bulky tolerance at the receptor site of the enzyme.

The most important findings in our study were

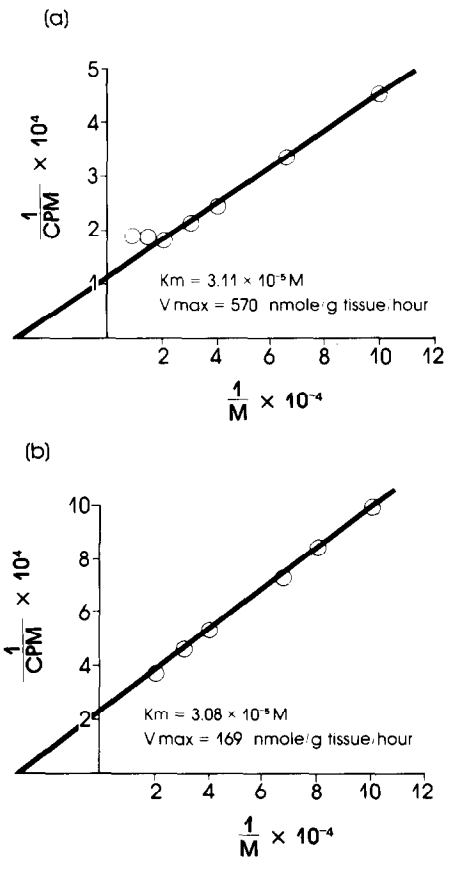



Fig. 4. Lineweaver–Burk plots of the *N*-methylation of SK&F 64139 with [$^{14}$C]SAM and the partially purified dog liver *N*-methyltransferase. Panel a: SAM, 0.5 mM; panel b: SAM, 0.05 mM. Incubation was carried out at 37° for 15–30 min and was terminted by the addition of 0.5 M sodium borate (pH 10). The $^{14}$C-methylated SK&F 64139 was extracted into ethyl acetate twice. The combined extracts were evaporated to dryness and counted in Bray's solution.

that this dog liver *N*-methyltransferase exhibited very low $K_m$ and high $V_{max}$ values for SK&F 64139, and corresponding enzyme activity was totally absent in both rat and monkey liver. Some activity (25 nmoles per g tissue per hr) was detected in the mouse liver, but it was significantly lower than that in the dog liver. These observations provide an explanation for why *N*-methylation is the dominant metabolic pathway for SK&F 64139 in dog, but not in other species [5].

Similar species differences in metabolism might also be expected for other arylalkylamine-type compounds, which could possibly result in different detoxication and bioactivation effects. Furthermore, SK&F 29661, a very poor substrate for the enzyme, was found to be excreted unchanged in dog urine (unpublished data), suggesting a good correlation between the *in vivo* and *in vitro* metabolism of the compound.

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