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Pineal Hydroxyindole-O-Methyltransferase: Mechanism, and Inhibition by Scotophobin A

NOBUHIRO SATAKE AND BRUCE MORTON

Department of Biochemistry and Biophysics
John A. Burns School of Medicine
University of Hawaii, Honolulu HI 96822

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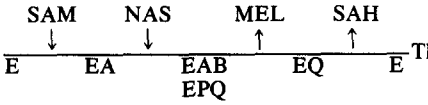
SATAKE, N AND B MORTON *Pineal hydroxyindole-O-methyltransferase Mechanism, and inhibition by scotophobin A* PHARMAC BIOCHEM BEHAV. 10(4) 457–462, 1979.—We had shown that the behaviorally active peptide, scotophobin A, a synthetic analogue of native scotophobin, acted to increase dark avoidance in goldfish by inhibiting pineal hydroxyindole-O-methyltransferase (HIOMT), the enzyme which converts N-acetylserotonin (NAS) to melatonin (MEL) Here we determine the reaction sequence of bovine pineal HIOMT and the mechanism whereby scotophobin A inhibits this enzyme Initial rate studies in which the substrates NAS and the methyl donor, S-adenosylmethionine (SAM), were independently varied indicated the enzyme reacted by a sequential mechanism With the product, S-adenosylhomocysteine (SAH), included in the reaction mixtures, data were obtained consistent with the following order of substrate addition and



The turnover number was 5 7 moles melatonin formed/mole HIOMT/min The substrate K_{Ms} were $4\,2\times10^{-4}$ M for NAS and $4\,9\times10^{-5}$ M for SAM Further studies showed that scotophobin A is an inhibitor ($K_I=7\times10^{-7}$ M) competitive with NAS, indicating that this peptide combines with the enzyme-SAM complex The structural similarity of the tyrosinamide end of scotophobin A to NAS and several other HIOMT inhibitors, including two antischizophrenic drugs, is consistent with these observations

Hydroxyindole-O-methyltransferase mechanism Scotophobin mechanism Pineal gland and scotophobin
Scotophobin inhibition of melatonin formation Scotophobin inhibition of HIOMT

IT HAS been reported that synthetic scotophobin A, a peptide closely related to native scotophobin originally isolated from the brain of rats trained with shock to avoid the dark [30], causes dark avoidance behavior in several species [2, 8, 10]. We showed that in goldfish, this, and other scotophobin-induced phenomena, required the presence of the pineal gland [27,28]. While studying the formation of pineal melatonin from serotonin, via N-acetylserotonin, we found that several conditions causing the elevation of pineal N-acetylserotonin *in vivo* also produced dark avoidance behavior in goldfish [28] It was further observed that the scotophobin-induced dark avoidance in fish was associated with the inhibition of N-acetylserotonin conversion to melatonin [28]. We then discovered that scotophobin directly inhibited pineal hydroxyindole-O-methyltransferase (HIOMT), the enzyme which catalyzes the conversion of N-acetylserotonin to melatonin [28]

Not long after the discovery of melatonin, reported in 1958 [24], HIOMT was identified and purified 25-fold from bovine pineal glands by Axelrod and Weissbach [1]. About a decade later bovine pineal HIOMT was purified in a different manner and partially characterized by Jackson and Lovenberg [20]. They found that their preparations of HIOMT reached apparent homogeneity after only 25-fold purification and had no higher specific activity than the Axelrod-Weissbach preparation [1]. This was apparently because HIOMT constituted four percent of the cytosol proteins of the bovine pineal. They also reported that HIOMT was a 79,000 dalton dimer with identical subunits which had a tendency to form larger aggregates [20]. Both HIOMT preparations exhibited the very low turnover number of about 0 4 moles melatonin formed/mole enzyme/minute

Shortly thereafter Karahasanoglu and Ozand presented a third quite divergent study of bovine pineal HIOMT [21]. Utilizing a new assay based upon the observations that the reaction product, S-adenosylhomocysteine (SAH), was a potent product inhibitor and that oxaloacetate and bicarbonate activated the enzyme, they purified the enzyme an apparent 2800-fold They too reported the presence of HIOMT polymers beginning at 83,000 daltons, however some activity was associated with a 21,800 dalton fraction [21]. It is difficult to compare this latter study of HIOMT to the earlier studies because of several major differences in the assay conditions which resulted in quite divergent kinetic constants It may be possible that due to these differences, the purity of their final preparation was similar to that of the earlier workers

Regarding the inhibition of HIOMT, both p-chlo-

romercuribenzoate [1] and GSH [21] totally inactivated the enzyme, indicating the involvement of a sulfhydryl group at the active site. In addition a number of reports have appeared describing the inhibitory effect of a large series of synthetic organic compounds [13–18] The most potent of those synthesized [15] was not more inhibitory ($I_{50}=5\times10^{-6}$ M) than SAH ($K_i=2.1\times10^{-6}$) [7]

HIOMT has been shown capable of methylating several substrates other than N-acetylserotonin, including indoleamines and catecholamines, to produce psychoactive compounds implicated in mental illnesses [12] In keeping with this finding, two drugs used in the treatment of schizophrenia, haloperidol and oxypertine, were also found to inhibit HIOMT with I_{50} values of $4\ 6\times10^{-4}$ M and $7\ 8\times10^{-5}$ M, respectively [19]

HIOMT has long been recognized as an important control enzyme about which information regarding kinetic properties and inhibition sensitivity is needed [25]. In view of our observation [28] that the Ungar *et al* [29] synthetic analogue of scotophobin is one of the most potent inhibitors for the enzyme yet found ($I_{50}=6\times10^{-7}$ M), we decided to begin a series of studies designed to further characterize the enzymatic and inhibitory properties of pineal HIOMT Here we define the reaction sequence of the enzyme, and determine the molecular basis of scotophobin inhibition

METHOD

Biochemicals

Scotophobin A [29] was generously supplied by the late Georges Ungar, who indicated it to be a pure synthetic analogue possessing 25% of the activity of native scotophobin For another view on the structure of the scotophobins, see Guttman *et al* [11] N-Acetylserotonin (NAS), S-adenosylmethionine (SAM) and S-adenosylhomocysteine (SAH) were purchased from Sigma Chemical Company Bovine pineal glands were from Pel Freeze Inc

HIOMT Assay

The enzyme was assayed by measuring the formation of melatonin from N-acetylserotonin and S-adenosylmethionine The triplicate 1 ml incubation mixtures within 15 ml Corex tubes contained 0.1 M potassium phosphate, pH 7 9, various indicated concentrations of NAS, SAM, SAH and scotophobin in water and 1.36 μg of the above HIOMT preparation After 30 minutes incubation at 37°C, the reactions were stopped by the addition of 4 ml chloroform. Melatonin was extracted into the chloroform by 30 seconds of shaking and the aqueous phase discarded by aspiration After the chloroform was washed twice with 3 ml deionized water, it was removed by drying The residue remaining was dissolved in 1 ml of water and the chloroform extraction repeated. About 100% of added melatonin was recovered by this procedure, compared to less than 0.001% of NAS and SAM The extracted melatonin in 1 5 ml of deionized water was measured fluorometrically with an Aminco-Bowman spectrofluorometer (excitation, 290 nm, emission, 360 nm) The fluorescence reading was proportional to melatonin concentration over the 0–100 ng range employed.

HIOMT Purification

The procedure used was that of Axelrod and Weissbach [1] which has been reported to result in essentially homogenous HIOMT [9] Bovine pineal glands (16 g) were homogenized by three passes with a large motor driven Potter-Elvejhem homogenizer in 80 ml of 0 154 M KCl The homogenate was centrifuged (78,000 G for one hour) and 52 ml of the supernatant solution was adjusted to pH 6.5 with 1 N KOH and centrifuged for 10 min at 8,000 G. Saturated ammonium sulfate solution (pH 8 0) was added to the 50 ml supernatant solution to produce a 45% saturation After centrifugation (10 min at 8,000 G), the supernatant solution was removed and brought to 68% saturation The precipitate from a further similar centrifugation was dissolved in 12 ml deionized water and adjusted to pH 5.3 with acetic acid. Two ml of alumina gel Cγ (35 mg) was added to this solution which was then stirred gently for one minute and centrifuged (8,000 G for 5 min). After centrifugation the gel was washed with 5 ml water and the HIOMT eluted by washing the gel with three 5 ml portions of 0.1 M potassium phosphate, pH 6 5 The last two eluates were used for the enzyme assay The protein concentration of this preparation was 136 μg/ml The specific activity was 1 2 nmoles melatonin formed/mg protein/ml at 37°C in standard assay mixtures containing $1\ 15\times10^{-5}$ M SAM and 10^{-5} M NAS.

RESULTS

Bovine pineal hydroxyindole-O-methyltransferase catalyzes the reaction of two substrates, the methyl donor, S-adenosylmethionine, and the favored indoleamine, N-acetylserotonin, to form S-adenosylhomocysteine and melatonin We first wished to determine which of the several possible bisubstrate catalytic mechanisms this enzyme employed [3] The initial velocity patterns presented in Fig 1 indicated a reaction mechanism of the sequential type [3] in which the two substrates must be bound before the products are released The double reciprocal plots, each representing a different concentration of the nonvaried substrate, converge to the left of the ordinate axis indicating the changes in both slope and intercept values Such is not the case in double displacement (ping pong) mechanisms which give rise to parallel patterns showing changes in intercept values only [3]

Computer-assisted multiple regression analyses of the data points generating the plots of Fig 1 were performed to evaluate them both in terms of slope and intercept There were no systematic changes in the distribution of the data which would influence the validity of the usage of this type of data treatment The analysis showed a significant difference among slopes, $t(71)=6\ 29$, $p<0\ 01$, and among the intercepts on the vertical axis, $t(71)=4\ 00$, $p<0\ 01$.

A replotting of the apparent maximum velocity data of Fig 1, according to the method of Florini and Vestling [9], is shown in Fig 2. From the reciprocal of the ordinate and abscissal intercepts, respectively, we were able to evaluate V_{max} (71 nmoles melatonin formed/mg/min), K_M for NAS ($4\ 2\times10^{-4}$ M) and K_M for SAM (4.9×10^{-5} M). The turnover number was 5.7 moles melatonin formed/mole of HIOMT/minute

We next sought to determine the order of substrate addition and product release for HIOMT. Tm do this we measured initial rates in the presence of the product, S-adenosylhomocysteine At nearly saturating levels of SAM ($4\ 6\times10^{-5}$ M) with NAS as the varied substrate, the addition of $1\ 04\times10^{-6}$ M of SAH had no inhibitory effect (Fig. 3A) An analysis of rate measurements in the presence

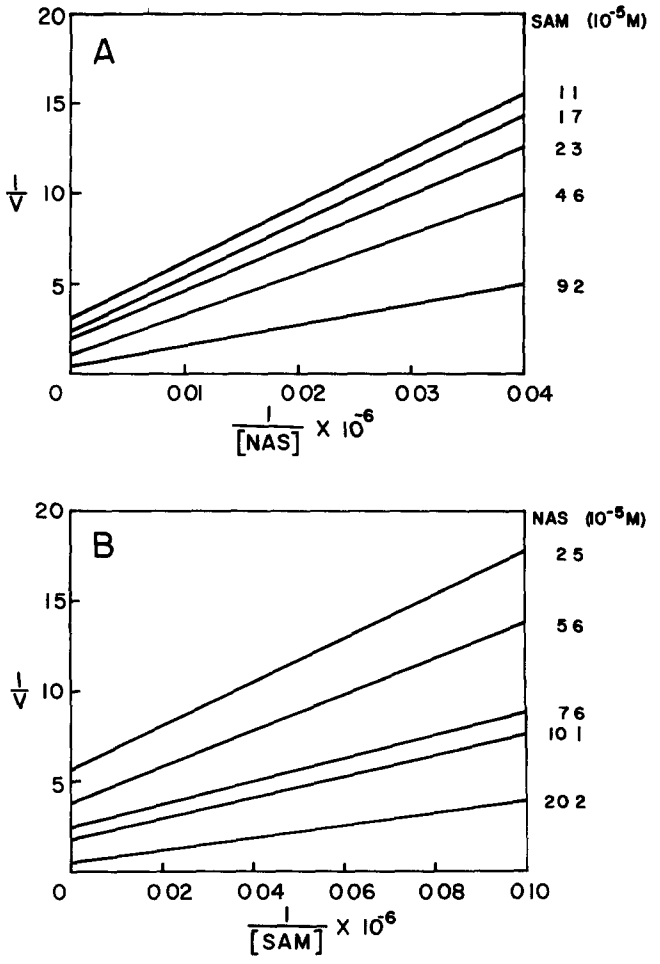


FIG 1 Double reciprocal plots of the effect of substrate concentration upon HIOMT activity Plots were computer generated by the method of least squares. The fit of the plots to the data points was good (r^2 values>0 8) $1/V = 1/\text{melatonin} \times 10^{-6}$

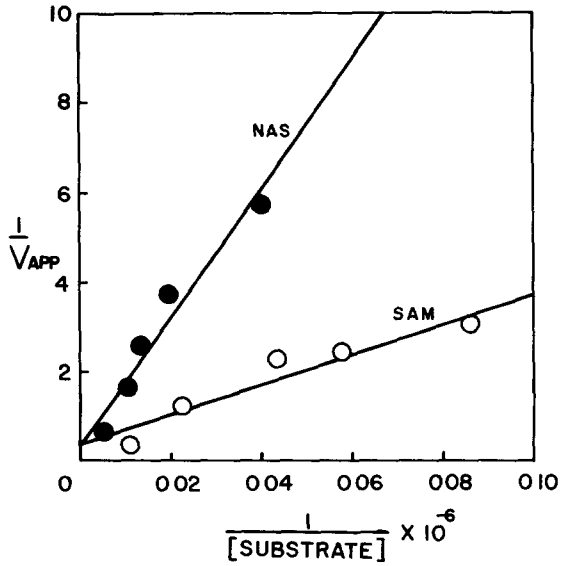


FIG 2 Secondary plots of $Vapp^{-1}$ vs $[\text{substrate}]^{-1} \times 10^{-6}$

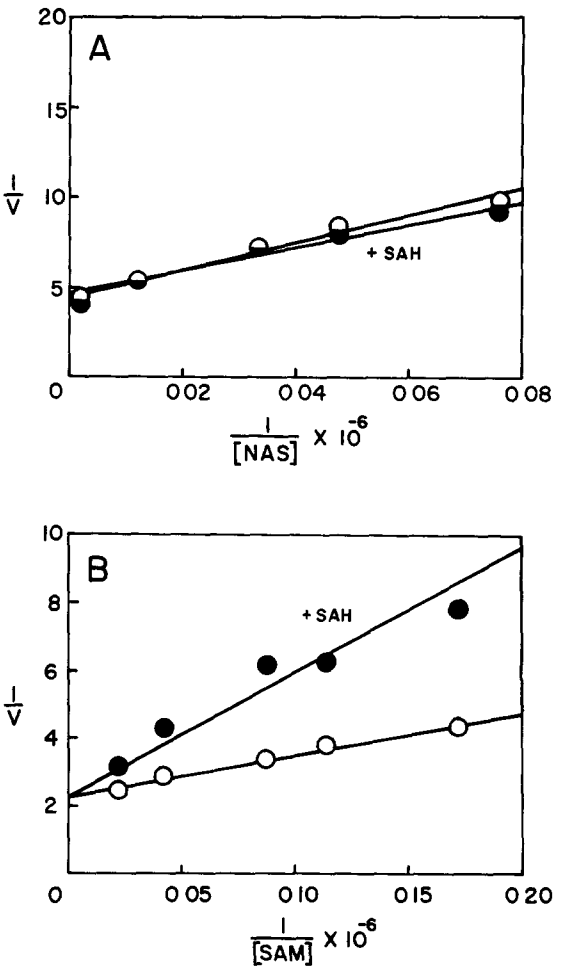
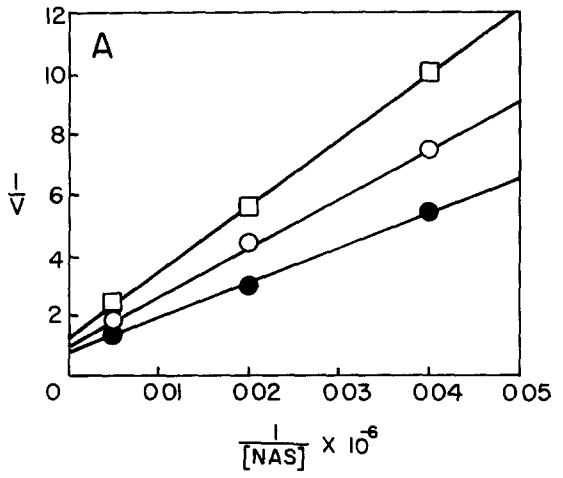


FIG 3 The effect of the product, 1 μM SAH, upon the activity of HIOMT in the presence of varied substrate levels ○=control, ●=plus SAH, $1/V = 1/\text{melatonin} \times 10^{-6}$

and absence of SAH showed no significant difference between the two ($t<1.0$) However, SAH at the same concentration was an inhibitor competitive with SAM in the presence of saturating levels of NAS ($1\ 83 \times 10^{-4}$ M) (Fig. 3B). An analysis indicated these SAH data were significantly different (slope effect: $t(29)=3.61$, $p<0.01$; intercept effect: $t(29)=1\ 95$, $p>0.1$) These product inhibition patterns were consistent with an Ordered Bi Bi mechanism [3, 4, 5] where SAM is bound before NAS and SAH is released last after melatonin This reaction mechanism can be depicted as follows

We next investigated the kinetics of the scotophobin A inhibition of HIOMT. In Fig 4A it may be seen that the addition of scotophobin A (2.8 and 5.6×10^{-7} M) produced competitive inhibition when SAM was held constant at a saturating level (2×10^{-4} M) and NAS varied. Analyses



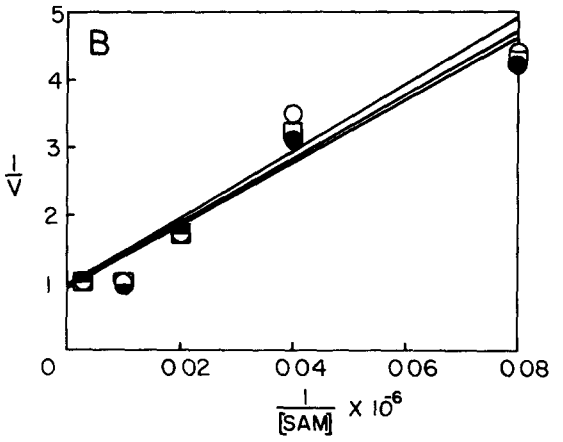


FIG 4 The effect of scotophobin upon HIOMT activity in the presence of various concentrations of substrates and products A=2×10^{-4} M SAM, B=2×10^{-4} M NAS, ●=control, ○=plus 2.8×10^{-7} M scotophobin, □=plus 5.6×10^{-7} M scotophobin, 1/V=1/melatonin$\times10^{-6}$

showed a significant slope effect, $t(25)=13.6$, $p<0.01$, but no intercept effect, $t<1.0$ When NAS was held constant at 2×10^{-4} M and SAM varied (Fig. 4B) the addition of 2.8 and 5.6×10^{-7} M scotophobin A produced no enzyme inhibition, $t<1.0$ for both slope and intercept The dissociation constant (K_I) for scotophobin A under these conditions was calculated [4] from the horizontal axis intercepts of the control curve and the scotophobin curves of Fig 4A to be about 7×10^{-7} M

In Table 1, derived from Cleland [5,6], it may be seen that the data presented in Fig. 4 are most consistent with binding by scotophobin to the E-SAM form of HIOMT. To further clarify which form of the enzyme was being poisoned by scotophobin A, Table 1 suggested that inhibition studies of the enzyme at nonsaturating concentrations of NAS with SAM variable would be appropriate. Under these conditions (NAS was 2.3×10^{-5} M), an uncompetitive inhibition by scotophobin A resulted (Fig 5), confirming that indeed this peptide inhibitor combined with the E-SAM form of HIOMT (slope effect $t(29<1.0$; intercept effect· $t(29)=2.62$, $p<0.02$)

TABLE 1

PATTERNS PRODUCED BY DEAD END INHIBITORS

Inhibitor Binding Site	Non-Varied Substrate: At Saturation		Non-Varied Substrate: Below Saturation	
	Varied Substrate: A	B	A	B
E	C	—	C	N
EA	—	C	U	C
EAB	U	U	U	U
EQ	U	U	U	U

C, N and U refer to competitive, noncompetitive and uncompetitive inhibition A and B are the first and second substrates bound respectively P and Q are the first and second products released

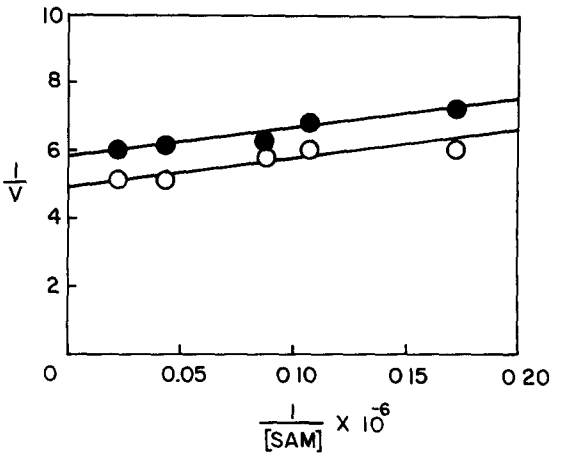


FIG 5 The effect of scotophobin upon the activity of HIOMT in the presence of nonsaturating levels of substrates ○=control (2.3×10^{-5} M NAS), ●=plus 2.8×10^{-6} M scotophobin, 1/V=1/melatonin$\times10^{-6}$

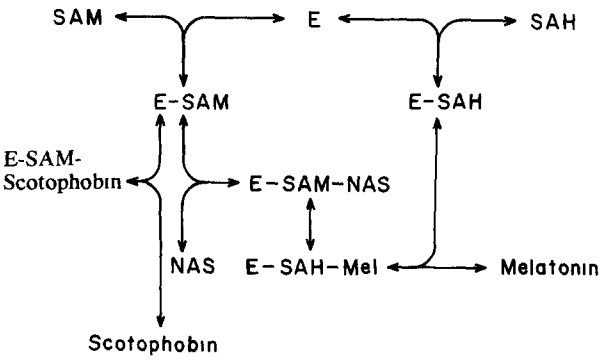


FIG 6 Diagram of the reaction mechanism for the enzyme, HIOMT, and its inhibition by scotophobin

The steps of the reaction catalyzed by HIOMT and the point of scotophobin inhibition, as determined here, are shown in Fig 6 Free HIOMT first binds with S-adenosylmethionine to form an E-SAM complex. Both the second substrate, N-acetylserotonin, and scotophobin A

compete for the E-SAM complex Successful binding of NAS to the EA complex leads to methyl group transfer, followed by the release of the first product, melatonin S-Adenosylhomocysteine dissociates last to regenerate free HIOMT

DISCUSSION

As a result of these studies, bovine pineal hydroxy indole-O-methyltransferase was found to function via a reaction sequence of the Ordered Bi Bi type The Ping Pong Bi Bi reaction type where the enzyme forms a covalently intermediate was eliminated as a possibility because initial velocity measurements did not produce double reciprocal plots of constant slope, but rather generated plots with varying slope. That the sequential mechanism was ordered and not random with respect to substrate addition was confirmed by the observation that the product, SAH, did not compete with both substrates, but only with SAM.

Some variations are present in the kinetic constants generated by the four major studies, including this one, of highly purified HIOMT [1, 19, 20] Values for V_{max} were determined only by ourselves and by Karahasanoglu and Ozand [21] They were 71 and 200 nmoles melatonin/mg HIOMT/ min, respectively From these values the turnover number was computed to be 5 7 and 16 moles melatonin/mole HIOMT/min, respectively, assuming a molecular weight of 80,000 Although these values are very low, they are not as low as the 0.44 moles melatonin/mole HIOMT/min value approximated by Jackson and Lovenberg [20] from their own and Axelrod and Weissbach's [1] specific activity data In view of such catalytic inefficiency it is not a surprise that four percent of all pineal cytosol proteins should be HIOMT [20] The reported K_M values for the substrates of HIOMT are also quite variable This is in part because they are K_{Mapp} values except for those determined here. Thus, for NAS the K_M varies from $5\ 9\times10^{-6}$ M [21] through $4\ 6\times10^{-5}$ M [1] to $4\ 2\times10^{-4}$ M, reported here Similarly the K_M for SAM varies from $2\ 3\times10^{-7}$ [21] to $5\ 4\times10^{-5}$ M [1] and $4\ 9\times10^{-5}$ M, also reported here. The addition of activators and the partial avoidance of product inhibition in the assay may account for the lowest of the reported K_M values [21].

Regarding the order of substrate addition and product release for HIOMT, the product inhibition studies with one substrate varied and the other at saturation produced definitive data The presence of SAH was not inhibitory when SAM was saturating and NAS was varied However when NAS was saturating, SAH was a competitive inhibitor to varied SAM This not only indicated that SAM and SAH competed for the same form of HIOMT but also, according to the analysis by Cleland [3–6], SAM was the first substrate on HIOMT and SAH the last product off If NAS had been the first substrate and melatonin the last product, the SAH inhibition patterns would have been uncompetitive against varied NAS and noncompetitive against varied SAM [3–6]

Regarding the mechanism of scotophobin A inhibition of HIOMT this behaviorally active peptide effectively inhibited the enzyme ($K_I=7\times10^{-7}$ M) except under conditions where SAM was limiting and SAH levels were elevated This evidence indicates scotophobin A and NAS compete for the E-SAM form of the enzyme Such a conclusion is reinforced by the observation that the COOH end of scotophobin A terminates in -glycine-tyrosineamide, a peptide which can be visualized to have the same configuration as NAS The most effective of inhibitors synthesized by Ho and coworkers [13–19] also are structurally related to NAS, as are the antischizophrenic agents, haloperidol and oxypertine which also inhibit HIOMT [19]

The discovery that HIOMT can be effectively inhibited by a peptide has opened several interesting avenues of research involving the effect of natural and synthetic peptides upon this important enzyme Regarding the control of the diurnal variation in the activity of HIOMT [26], it appears that although biosynthesis-degradation [26], aggregation-disaggregation [20] or substrate-product level [9] mechanisms may play a role, endogenous peptides may also significantly alter the activity of this enzyme. Inappropriate activity of HIOMT or enzymes of nearby reaction pathways might lead to elevations in the production of endogenous hallucinogens such as harmine-like compounds [22,23] or methylated catecholamines [12] This could conceivably result in altered mental states such as those present in schizophrenia [12] or possibly those causing scotophobin-induced dark avoidance

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