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# Scotophobin A Causes Dark Avoidance in Goldfish by Elevating Pineal N-Acetylserotonin

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SATAKE, N AND B E MORTON *Scotophobin A causes dark avoidance in goldfish by elevating pineal N-acetylserotonin* PHARMAC BIOCHEM BEHAV **10**(4) 449–456, 1979 —We had shown that synthetic rat scotophobin A caused several effects upon goldfish, apparently mediated by the pineal gland Here we report that norepinephrine decreased goldfish dark avoidance in a manner that was blocked by scotophobin or pinealectomy Increased dark avoidance was caused by either propranolol or scotophobin alone Certain components of the pineal melatonin pathway also affected goldfish light-dark preference serotonin, and especially N-acetylserotonin, increased dark avoidance, as did the hydroxyindole-O-methyl-transferase (HIOMT) product inhibitor, S-adenosyl-homocysteine Melatonin and S-adenosyl-methionine were without effect in this regard Pinealectomy prevented the dark avoidance increase caused by serotonin and N-acetylserotonin These data suggested that increased dark avoidance behavior in goldfish was correlated with N-acetylserotonin buildup in the pineal, and that scotophobin could cause this, if it were to inhibit pineal HIOMT To test this hypothesis the effect of various agents upon pineal melatonin levels was determined Scotophobin was found to both reduce pineal melatonin and to block the melatonin-increasing effect of N-acetylserotonin This led to the discovery that, indeed, scotophobin was an effective inhibitor ($K_{I_{50}}$, $6\times10^{-7}$ M) of purified bovine HIOMT

Scotophobin Pineal gland Dark avoidance N-acetylserotonin Hydroxyindole-O-methyl-transferase

SYNTHETIC scotophobin A is a peptide closely related to native scotophobin [17] which was isolated from the brain of rats trained with electric shock to avoid the dark [18] We have reported that this scotophobin-like peptide [8] caused several effects upon goldfish which required the presence of the pineal gland [15] These include an increased sensitivity to shock, an increase in dark avoidance (also reported by others; [5,7]), and the prevention of norepinephrine-induced chromatophore aggregation. Here, we inquire whether norepinephrine might also cause a pineal gland-dependent light-dark behavioral effect, and if so, whether such might also be abolished by scotophobin

A major function of the pineal gland is associated with the formation of melatonin from serotonin via N-acetylserotonin (NAS) [2]. The latter is methylated to form melatonin by hydroxyindole-O-methyl-transferase (HIOMT) This is brought about at the expense of the methyl donor, S-adenosyl-methionine (SAM), which is converted to S-adenosyl-homocysteine (SAH), a product inhibitor of HIOMT. In view of the association of the pineal gland with the effects produced by scotophobin, we wished to test several of these pineal metabolites upon goldfish light-dark behavior. An interesting pattern has emerged both suggesting that an elevation of N-acetylserotonin was associated in all cases with increased dark avoidance behavior and that scotophobin must be an inhibitor of HIOMT. The results of our experiments supported this hypothesis and are presented here

## METHOD

### *Biochemicals*

Synthetic scotophobin A [17] was generously supplied by the late Georges Ungar who indicated its activity to be 25% of native rat scotophobin in the mouse assay. When needed, a portion of the powder, stored at –20°C, was weighed out, dissolved in water and frozen Each of these was utilized within two months. Norepinephrine bitartrate, serotonin oxalate, propranolol, N-acetylserotonin, melatonin, S-adenosyl-methionine and S-adenosyl-homocysteine were purchased from Sigma Chemical Company The dosages employed were based upon semi-empirical decisions, due to the absence of dose response data in some cases Bovine pineal glands were from Pel Freeze, Inc

### *Fish*

The experimental animals used in these studies were common goldfish *(Carassius auratus)* originally supplied by Ozark Fisheries. The goldfish were between 7.5 and 8 cm in length. After about a month in a large holding tank, they were separately maintained in two gallon tanks whose water was filtered, aerated and kept about about 18°C. The fish were each fed two Purina koi pellets at 5 p m daily and illuminated from 8 a m to 8 p.m. under incandescent light. All experiments were carried out between about 10 a m. and 3 p.m

Scotophobin and other agents, dissolved in 0 9% NaCl, pH 7 4, were administered to the fish by way of 10 $\mu$l intracranial injections using a 25 $\mu$l Hamilton microsyringe with a No 30 ga needle and a guard set at 3 mm to prevent insertion beyond the supracerebral space [1]

### *Pinealectomy*

When it became appropriate to determine the involvement of the pineal gland in the action of scotophobin,

goldfish were pinealectomized under the anesthetic ethyl-m-aminobenzoate (Tricaine, 1/8,000 dilution) The pineal glands were exposed by making a circular cut in the parietal bone (less than 5 mm in dia ) such that the center of the circle bisected an imaginary line connecting the posterior margins of the orbital cavities Under a dissecting microscope (20 magnifications) the pineal gland was removed with a forceps After the operation, the fish were kept in their home tanks for a month to permit recovery and the resealing of the skull opening Post-experimental inspection of these fish verified that no pineal gland regeneration had occurred In sham operations all steps were carried out except the gland was not excised

*Light and Dark Avoidance Behavior Assay*

*Equipment* Light preference behavior (light or dark avoidance or escape) was produced and measured in wooden shuttleboxes that measured 52×18×15 5 cm deep. Each box was bisected across the center by a partial barrier 5 cm high that stopped 2 5 cm from the water's surface. Fish movement (response) across this barrier was monitored by a pair of photocells, 4 5 cm apart, placed upon the sides of the box 1 5 cm above the barrier A white light (stimulus) could be presented at either end of the box through frosted glass panels containing a 7 W Christmas tree light

*Experimental Procedure*

In order to determine the normal baseline light-dark preference of the individual fish and to establish their basal swimming activity levels, each animal was monitored in a daily baseline session for 4 days using the shuttleboxes Each baseline session consisted of 60 trials of 1 min dark or light avoidance measurement and 60 trials of 1 min of lights off general activity measurement An intertrial darkness interval of 10 sec separated the alternating measurement periods consisting of 1 min light or dark avoidance measurement and 1 min general activity measurement In the avoidance trial periods, one chamber of the shuttlebox was always darkened For example in the dark avoidance studies, it was the side of the shuttlebox occupied by the fish, while in the light avoidance studies it was the chamber opposite the fish Crossing the barrier automatically tripped the photocells and switched the lighting to maintain the same light-dark relationship to the animal

After the basal light-dark preference and swimming activity levels were determined for the set of fish involved in an experiment, the fish were sorted into groups in such a way that the average basal activities of each group were as close to the other as possible Then the fish of each group were injected with scotophobin, saline or other agents and tested immediately and on subsequent days for their dark or light avoidance responses and for general activity in exactly the same way as baseline studies above Responses were monitored for every trial and recorded every ten trials (5 dark or light avoidance trials and 5 general activity trials) Statistical analysis was done using the recorded responses from these 5-trial segments as one measurement, unless otherwise noted Graphs were plotted in terms of mean responses of two segments per min (10 trials)

*Melatonin Assay*

The procedure used for the measurement of melatonin was derived from Axelrod and Weissbach [4] At the indicated times goldfish were sacrificed by decapitation and their heads immediately frozen with dry ice Their pineal glands were removed as described above and individually homogenized in 1 ml of phosphate buffer (0 05 M, pH 7.9) in a 10 ml hand operated Potter Elvejhem homogenizer The homogenates were transferred to 15 ml Corex centrifuge tubes, 4 ml of chloroform added and the tubes manually extracted for 30 sec The aqueous phase was removed by aspiration and the chloroform extract washed twice with 3 ml of deionized water. It was then dried with warm air and the remaining residue was dissolved in 1.5 ml deionized water

Melatonin was measured fluorometrically with an Aminco-Bowman spectrofluorometer (excitation, 290 nm, fluorescence, 360 nm) The fluorescence reading was proportional to melatonin concentration over the 0–100 ng range employed The UV-visible spectra of the extract was identical to that of commercial melatonin The recovery of melatonin added to the pineal homogenate was constant at about 40%

*HIOMT Purification*

The procedure used was derived from that of Axelrod and Weissbach [4]. Bovine pineal glands (16 g) were homogenized with a large Potter-Elvejhem homogenizer in 80 ml of 0 154 M KCl The homogenate was centrifuged (78,000 g for 1 hr) 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 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 stirred gently for 1 min 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 (Lowry assay [12]) of this preparation was 136 μg/ml. The specific activity under the conditions of our assay was 1 2 nmoles melatonin formed/mg protein/min at 37°C

*HIOMT Assay*

The enzyme was assayed by measuring the formation of melatonin from N-acetylserotonin and S-adenosyl-methionine The 1 ml incubation mixture within 15 ml Corex tubes, contained 0 1 M potassium phosphate, pH 7.9, $10^{-5}$ M NAS, 1 $15\times10^{-5}$ M SAM, various concentration of scotophobin and 1 36 μg of the above HIOMT preparation tion After 30 min incubation at 37°C, the reactions were stopped by the addition of 4 ml chloroform Melatonin was extracted into the chloroform by 30 sec 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 a 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 ml of deionized water was fluorometrically measured as described above

## RESULTS

### *Norepinephrine Effects on Goldfish Light-Dark Preference Behavior*

We had shown that in goldfish norepinephrine caused chromatophore aggregation [15] This was suppressed by scotophobin, provided that the pineal gland was present. In addition we verified that scotophobin caused pineal gland-dependent dark avoidance behavior in goldfish. These observations led us to inquire whether norepinephrine might also affect goldfish light-dark preference behavior. To test this, dark avoidance trials were run with 19 fish as follows· ten fish were injected with 3 $\mu$g of norepinephrine, the rest with saline. During two hours immediately following the injections, the fish were tested for their responses in the same manner as they were in the baseline sessions prior to injection (1 min dark avoidance trial followed by 1 min trial for general activity in darkness alternating for 2 hr). The results for these trials are shown in Fig. 1. The norepinephrine injected goldfish crossed into the lighted side of the shuttlebox about one half as often as the saline injected fish did, yet the general activity levels of the two groups were almost identical An analysis of variance was carried out using the scores of 12 five-trial segments for each response. An analysis showed this reduction in dark avoidance caused by norepinephrine to be significant· groups×two response measures (dark avoidance responses and general activity)×trials, $F(11,187)=2.34$, $p<0$ 02 Subsequent analysis of general activity levels of the two groups did not show any effect ($F_s<1$ 0). These results are opposite in direction to the increased dark avoidance behavior caused by scotophobin

To verify the action of norepinephrine upon light-dark preference behavior of goldfish, a light avoidance experiment utilizing 35 goldfish, divided into four groups was run The first group (N=11) was injected with 3 $\mu$g each of norepinephrine. The effect of the catacholamine $\beta$-pathway antagonist, propranolol, (17 2 $\mu$g each) (N=11) upon goldfish light-dark preference behavior was also tested. The third group (N=6) received both 3 $\mu$g norepinephrine and 0 1 $\mu$g scotophobin. The fourth group (N=7) were the saline injected controls Light avoidance trials of 1 min duration alternating with general activity trials of equal length were run during the 2 hr immediately following the injections The results, shown in Fig. 2A indicate that, indeed, norepinephrine caused increased light avoidance (reduced dark avoidance) behavior in the fish By contrast, both scotophobin plus norepinephrine, and propranolol alone acted in the opposite direction to increase dark avoidance behavior.

A summary of the analysis of variance showed a significant difference between NE and saline in groups×response measures×trials interaction, $F(11,176)=2.13$, $p<0.02$, but not in groups or groups×response measures effect ($F_s<1.0$). Comparison between NE and NE plus scotophobin showed significant interactions in groups×response measures, $F(1,15)=5.09$, $p<0.05$, and in groups×response measures×trials, $F(11,165)=2.01$, $p<0$ 05, but not in groups effect, $F(1,15)=2.35$, $p>0.14$. Comparison between propranolol and saline showed a significant groups×response measures×trials interaction, $F(11,176)=2$ 13, $p<0.05$, but not significant effects in groups or groups×response measures ($F_s<1$ 0).

Subsequent analysis of variance (groups×trials, based on light avoidance responses), showed a significant difference

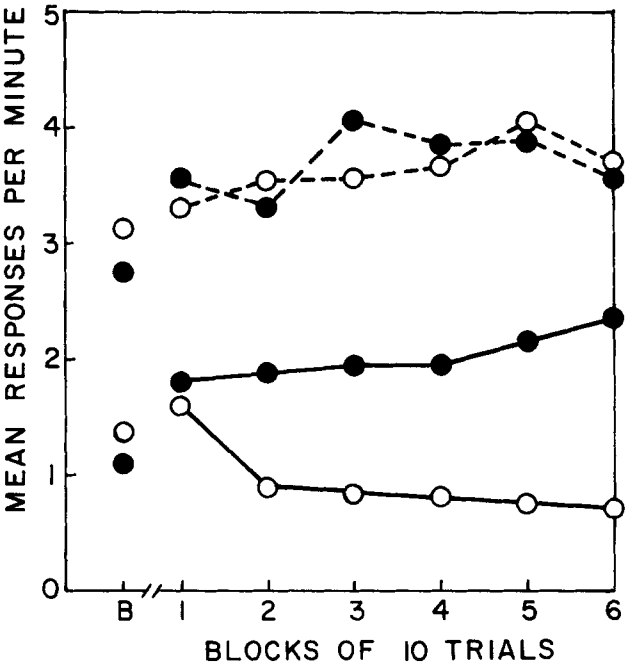


FIG 1 The effect of norepinephrine upon the dark avoidance behavior of goldfish The two groups of subjects were injected immediately before the first block of 10 trials as follows saline (●) and 3 $\mu$g norepinephrine (○) Their avoidance responses is plotted in solid lines while their activity in the dark is plotted in dashed lines Data points at B represent the mean of four days baseline sessions for both general activity (upper data points) and dark avoidance (lower data points) Since there was no significant heterogeneity of variance in this and the following experiments, only the average SEM's are presented The average SEM for this experiment was about 0 32

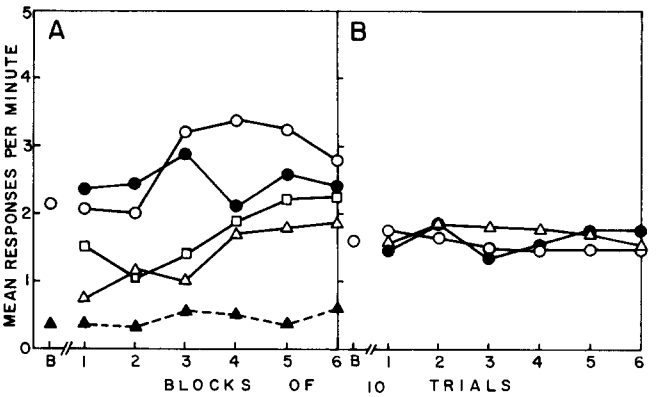


FIG 2 The effect of norepinephrine upon light avoidance behavior in goldfish (A) The four groups of subjects were injected immediately before the first trial block with saline (●), 3 $\mu$g norepinephrine (○), 17 2 $\mu$g propranolol (□) or 3 $\mu$g norepinephrine plus 0 1 $\mu$g scotophobin (△) The solid lines represent light avoidance responses while the dashed lines depict general swimming activity in darkness Data points at B represent the mean of four days baseline sessions for both general activity (▲) and light avoidance responses (○) Average SEM was 0 35 (B) The three groups of pinealectomized subjects were otherwise treated as in (A) above They were injected with saline (●), 3 $\mu$g norepinephrine or 0 1 $\mu$g scotophobin (△) Average SEM was 0 2

over trials between the effect of norepinephrine injection and saline injection, $F(11,176)=2\ 94$, $p<0\ 002$, between the effect of norepinephrine and of norepinephrine plus scotophobin, $F(11,165)=2\ 44$, $p<0.008$, between the effect of propranolol and of saline injection, $F(11,176)=3.13$, $p<0.001$. The groups effect was not significant among those comparisons ($F_s<1\ 0$) The change in dark preference responses could not be explained by modification of general activity since the general activity levels among groups did not show any significant effect ($F_s<1\ 0$)

The pineal gland was required in order for norepinephrine to modify light-dark behavior in goldfish, as was also the case for scotophobin This was indicated in the experiment shown in Fig 2B where 24 pinealectomized fish divided into three groups (saline, 3 $\mu$g norepinephrine, and 0 1 $\mu$g scotophobin) were tested for light avoidance alone during the 2 hr immediately following the injections An overall analysis of variance showed no significant effects due to groups differences ($F_s<1\ 0$)

*The Effect of Pineal Melatonin Metabolites in Goldfish Light-Dark Preference Behavior*

Since these experiments made it clear that the light-dark preference behavioral effects of norepinephrine and scotophobin [15] each required the presence of the pineal gland in goldfish, inquiry was next directed toward the possible involvement of pineal function, namely melatonin biosynthesis, in goldfish light-dark preference behavior Melatonin is formed from serotonin via N-acetylserotonin (NAS) (Fig 3) The conversion of the latter to melatonin is catalyzed by hydroxyindole-O-methyl-transferase (HIOMT). This is accomplished in the presence of the methyl donor, S-adenosyl-methionine (SAM) which is converted to S-adenosyl-homocysteine (SAH) [2]

The effect of serotonin upon goldfish light-dark preference behavior is shown in Fig 4A In this experiment 5 goldfish were injected with 50 $\mu$g serotonin in 10 $\mu$l saline and 7 fish were injected with saline alone Light avoidance and the general activity trials were run during 2 hr immediately following The results indicated that serotonin, like scotophobin, increased dark avoidance The analysis of variance showed a significant difference between the effect of serotonin and saline injection (group×trials interaction), $F(11,121)=2\ 24$, $p<0\ 02$ General activity levels of two groups did not show any significant effect ($F_s<1\ 0$)

N-acetylserotonin (NAS) at one tenth the dosage used for serotonin also increased dark avoidance behavior This is shown in Fig 4B where 24 fish were used, half of which were injected with 5 $\mu$g NAS, the others receiving saline alone The change in light avoidance responses of the NAS group during the hour following the injection gradually increased with time even though the general activity level did not differ significantly from the saline injected group An analysis of variance showed a significant difference between the two groups over the time course of the experiment (group×stimuli×blocks), $F(5,110)=2\ 63$, $p<0.03$ There were no significant effects due to group differences in the general activity levels ($F_s<1\ 0$)

A similar light avoidance experiment, with general activity measures, run with 21 animals, 11 of which were injected with 4 $\mu$g melatonin, did not show any significant effect of melatonin upon the light-dark preference behavior of the goldfish (data not shown) ($F_s<1\ 0$) A second melatonin experiment employing a higher dosage level was run In that

BIOSYNTHESIS OF MELATONIN BY THE PINEAL GLAND

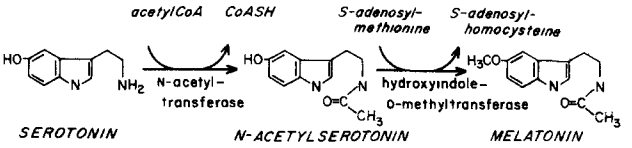


FIG 3 Biosynthesis of melatonin by the pineal gland

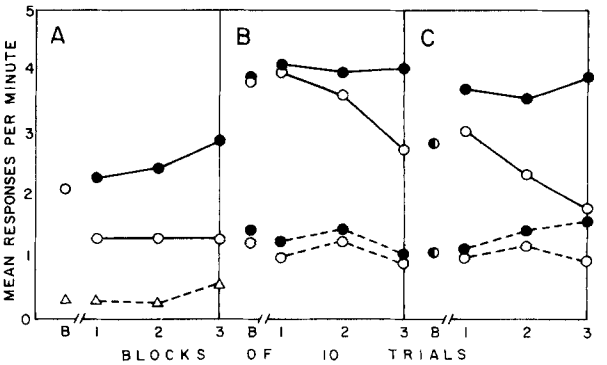


FIG 4 The effect of serotonin, N-acetylserotonin and S-adenosyl-homocysteine upon light avoidance behavior of goldfish (A) The two groups of subjects were injected with 50 $\mu$g serotonin (○) or saline (●) Average SEM was 0 33 In Fig 4A (only) data were plotted on the basis of blocks of 20 trials (B) The two groups of subjects were injected with 5 $\mu$g N-acetylserotonin (○) or saline (●) Average SEM was 0 12 (C) The two groups of subjects were injected with 10 $\mu$g S-adenosyl-homocysteine (○) or saline (●) Average SEM was 0 51 Solid lines represent light avoidance responses while broken lines depict general swimming activity in darkness Data points at B represent the mean of four days baseline sessions for both general activity (lower data points) and light avoidance reponses (upper data points)

experiment, employing 24 goldfish, both light and dark avoidance were tested instead of the usual light avoidance and general activity This procedure measured the response of subjects, previously given the usual four days of baseline sessions, to either 10 $\mu$g melatonin in 10 $\mu$l saline, containing 2% ethanol, or to the saline-ethanol alone (1 mg melatonin was dissolved in a drop of 95% ethanol and diluted to 1 ml in isotonic saline) Immediately after injection they were tested for their response in dark and light avoidance situations for 1 hr as in the baseline sessions It was observed (data not shown) that melatonin decreased the general activity of the fish, but again did not alter their dark-light preference Activity suppression of rats by melatonin has been reported by others [19] An analysis of variance showed a significant groups effect, $F(1,22)=6\ 15$, $p<0\ 0213$, but there were no significant differences between groups in terms of stimulus specificity (groups×stimuli $F(1,22)=2\ 54$, $p>0\ 126$; groups ×trials×stimuli $F<1\ 0$)

Similarly, in light avoidance trials with 18 goldfish, half of which were injected with saline, the rest with 10 $\mu$g

S-adenosyl-methionine, no difference in light-dark preference activity from the controls was produced by this intermediate involved in the formation of melatonin from serotonin by the pineal gland. An analysis of variance did not show any significant difference in groups effect ($F<1.0$), in groups × response measure interactions ($F<1.0$) or in groups × response measure × trials interactions ($F=1.05$)

However, when S-adenosyl-homocysteine, a product of the HIOMT-catalyzed reaction forming melatonin, was tested, it was found that it produced substantial dark avoidance behavior (Fig 4C) In this light avoidance-general activity experiment with 18 goldfish, one group was injected with 10 $\mu$g SAH just prior to testing. The other groups received saline only. An analysis of variance showed a significant difference between groups, $F(1,6)=4.8$, $p<0.044$ Subsequent analysis showed a significant difference in light avoidance between the groups, groups effect $F(1,16)=6\ 57$, $p<0\ 05$, groups × trials interaction. $F(5,80)=3.21$, $p<0.05$, but no significant differences between the general activity levels of the two groups ($F<1\ 0$)

To inquire whether injected serotonin or NAS acted only via the pineal gland or more directly upon the brain to produce their behavioral effect, these compounds were injected into pinealectomized fish in two experiments (data not shown). In the first experiment 12 pinealectomized fish were divided into two groups, one injected with saline and one with 50 $\mu$g serotonin These groups were then tested in light avoidance sessions Their light avoidance behavior, monitored over the following hour, indicated no significant group differences ($F<1\ 0$) In the second experiment (dark avoidance-general activity) with 14 sham operated and 16 pinealectomized goldfish, half of each group was injected with 5 $\mu$g NAS and half were injected with saline Although the sham operated fish injected with NAS increased their dark avoidance when compared to the control fish, the pinealectomized fish did not

An overall analysis of variance showed a significant interaction among drug treatments, operations, two response measures and trials, $F(5,130)=2\ 66$, $p<0.0026$. Subsequent analysis showed that there were no significant differences between two drug treatments in pinealectomized fish, drugs effect $F(1,14)=1.66$, $p>0\ 2$; drugs × two response measures: $F<1.0$, drugs × trials $F(5,70)=2.16$, $p>0.05$, but there was a significant effect of drug treatment in sham operated fish, drugs × two response measures $F(1,12)=4\ 81$, $p<0.05$, and drugs × two response measures × trials $F(5,60)=2\ 48$, $p<0.05$ It also showed that the significant drugs effect in sham operated fish was due to a difference in dark avoidance responses, drugs × trials $F(5,60)=4.53$, $p<0\ 0014$, and not due to the general activity levels, drugs effect. $F<1\ 0$; drugs × trials $F(5,60)=1\ 22$, $p>0\ 3$. These results indicated that increased light avoidance behavior produced by injection of serotonin or NAS was mediated by the pineal gland

Thus, serotonin, and at a lower dosage, N-acetylserotonin, increased dark avoidance behavior in goldfish while melatonin and S-adenosyl-methionine were inactive in this regard S-adenosyl-homocysteine, a product inhibitor of HIOMT that causes a buildup of N-acetylserotonin in vitro [6], also increased dark avoidance. These data indicated that pineal NAS was the probable agent increasing dark avoidance behavior in goldfish if not directly, possibly via serotonin buildup And that any inhibitor of HIOMT, thus, would be expected to increase dark avoidance by producing a backlog of NAS It also appeared possible that scotophobin might be such an inhibitor

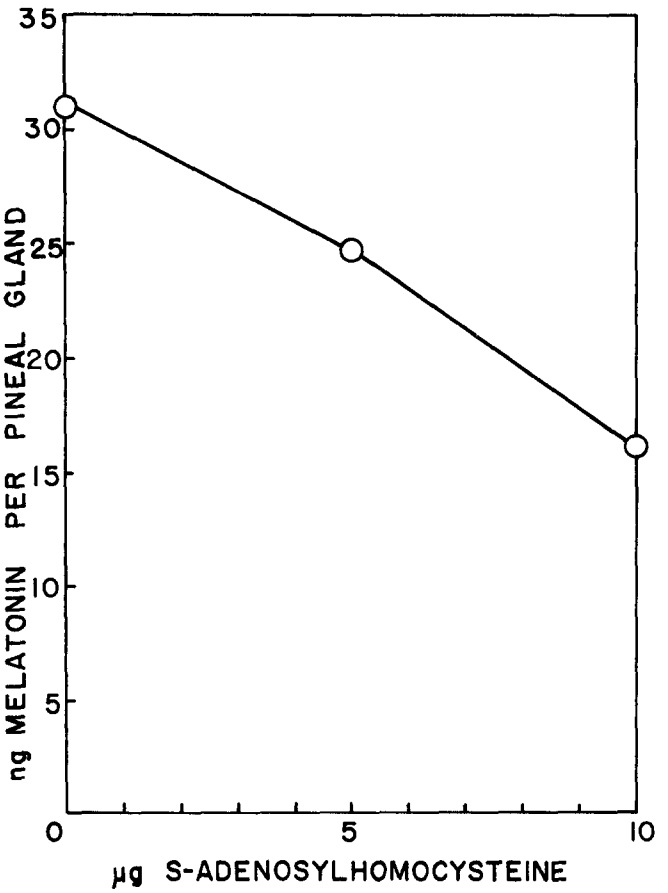


FIG 5 The effect of injected S-adenosyl-homocysteine upon goldfish pineal melatonin levels Average SEM was 1 73

*The Effect of Agents Modifying Goldfish Light-Dark Preference Behavior Upon Pineal Melatonin Content*

To test this hypothesis we directly measured the result of injecting goldfish with various agents upon their pineal melatonin levels. Shown in Fig. 5 is the effect of the HIOMT product inhibitor, S-adenosyl-homocysteine, upon the melatonin content of individual goldfish pineal glands (n=3 for each data point) The injection of 10 $\mu$g of SAH in 10 $\mu$l saline reduced pineal melatonin levels 1 hr later to about one half those of control fish receiving saline injections An analysis of variance showed a significant dosage effect, $F(2,6)=10\ 096$, $p<0\ 012$. Thus it was found possible to lower the levels of pineal melatonin by injecting an inhibitor of HIOMT

In order to evaluate whether scotophobin could also inhibit pineal HIOMT, 36 goldfish, divided into four groups, were injected (10 $\mu$l) with saline, 5 $\mu$g scotophobin, 5 $\mu$g N-acetylserotonin or 5 $\mu$g each of both NAS and scotophobin. The pineals of three fish from each group were then removed at 0, 30 or 60 min after the injection and their melatonin content determined. The results, shown in Fig. 6, indicated that by 1 hr scotophobin had lowered the melatonin content of the pineal gland by about one third In contrast, by 1 hr the melatonin content of the fish injected with NAS had almost doubled. The simultaneous injection of scotophobin with the NAS, however, not only suppressed

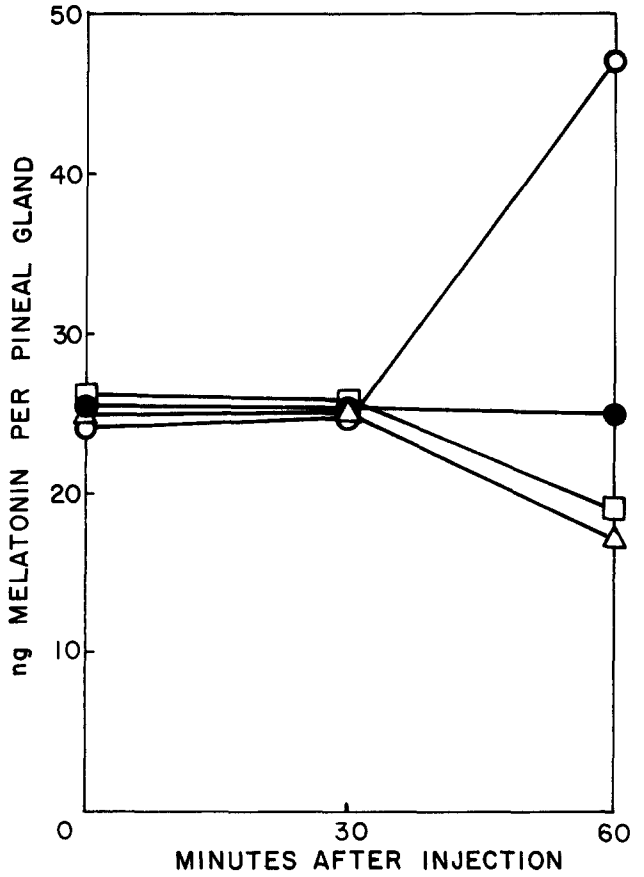


FIG 6 The effect of injected N-acetylserotonin and scotophobin upon goldfish pineal gland melatonin content The four groups of goldfish were injected with saline (●), 5 μg N-acetylserotonin (○), 5 μg scotophobin (△) or 5 μg N-acetylserotonin plus 5 μg scotophobin ([]) Average SEM was 1 24 ng

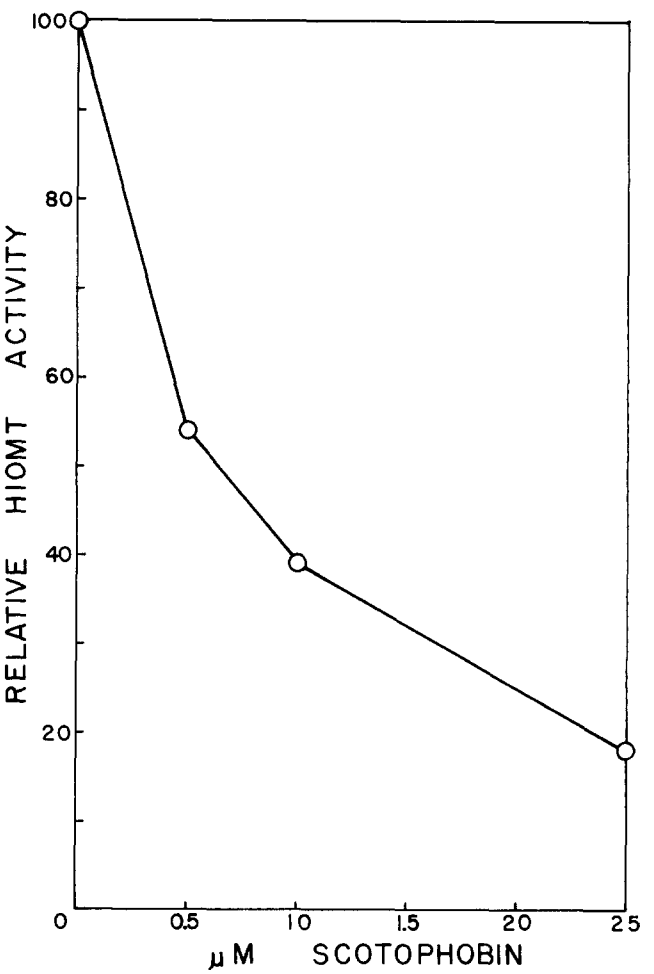


FIG 7 The effect of scotophobin upon the activity of purified bovine pineal hydroxyindole-O-methyl-transferase Average SEM was 6 98

any NAS induced increase in melatonin but also resulted in a drop in pineal melatonin to a level comparable to that caused by scotophobin injection alone

An overall analysis of variable showed significant groups effect, $F(3,24)=37\ 64$, $p<0\ 001$, and interaction between groups×sampling times, $F(6,24)=35.26$, $p<0\ 001$, but there was no significant effect of sampling times, $F(2,24)=2\ 83$, $p>0.07$. Subsequent analysis between the NAS and saline injected groups showed a significant group effect, $F(1,12)=26.99$, $p<0\ 001$, sampling times effect, $F(2,12)=29.31$, $p<0.001$ and groups×sampling times interaction, $F(2,12)=27\ 67$, $p<0\ 001$ Analysis between NAS and NAS plus scotophobin showed significant groups effect, $F(1,12)=64.98$, $p<0.001$, sampling times effect, $F(2,12)=27\ 67$, $p<0\ 001$ and groups×sampling times interaction, $F(2,12)=56\ 86$, $p<0.001$. There were also significant effects between scotophobin and saline injected groups, group effect $F(1,12)=11\ 16$, $p<0.006$, sampling times effect. $F(2,12)=8.69$, $p<0\ 005$; and groups×sampling times interaction: $F(2,12)=9.84$, $p<0.003$

*The Effect of Scotophobin Upon Pineal HIOMT Activity In Vitro*

To confirm whether scotophobin inhibited pineal HIOMT, this enzyme was purified from bovine pineal glands [4] and tested directly (Fig 7). Under the conditions of our assay ($10^{-5}$ M NAS, $1\ 15\times10^{-5}$ M SAM, 0.1 M potassium phosphate pH 7 9, 37°C), $6\times10^{-7}$ M scotophobin A reduced the activity of the purified bovine HIOMT by about 50% ($K_I=6\times10^{-7}$ M) while $25\times10^{-7}$ M scotophobin inhibited the enzyme about 80%. These concentrations were roughly comparable to those calculated to result in vivo by injecting goldfish with the doses of scotophobin utilized here, and those found in trained mice or those injected with scotophobin by Ungar [16].

## DISCUSSION

The present work reinforces our previous report indicating the essentiality of the pineal gland for scotophobin action [15]. Although the pineal gland was not implicated in the two studies on the binding distribution of labeled scotophobin within the brain of rodents [16] and fish [7], the difficulty of establishing the site of action of a compound from the results of such studies is well known.

In addition to scotophobin five new compounds were found here to influence goldfish light-dark behavior norepinephrine, propranolol, serotonin, N-acetylserotonin and S-adenosyl-homocysteine Two other pineal metabo-

lites, S-adenosyl-methionine and melatonin, were without activity in this regard, although melatonin exhibited its known depressing effect upon basal activity [19] These observations suggest that goldfish light-dark preference may be modulated by the balance of two pineal neurotransmitters: norepinephrine and N-acetylserotonin (or serotonin). Such binary control is common in many other systems under neuronal mediation

The norepinephrine-induced reduction in dark avoidance was shown to be specific rather than due to an enhancement of basal swimming activity or increased swimming in response to the light cycling occurring in the shuttleboxes. This was clearly shown both by the basal activity trials and by the reversal of response when the experiments were changed from dark avoidance to light avoidance. Like the effects of norepinephrine upon goldfish chromatophore expansion [15] scotophobin blocked the light-dark preference behavior effects of norepinephrine. Unlike the chromatophore system, the action of norepinephrine upon light-dark preference behavior required the presence of the pineal gland.

Specifically how norepinephrine excess might act to decrease dark avoidance behavior is not clear However, β-adrenergic receptors for catacholamines have been implicated in the function of the pineal gland [20] and propranolol, a specific blocker of such receptors, was found here to produce light-dark preference behavior opposite to that caused by norepinephrine. It is known that pineal serotonin, which here also opposed norepinephrine by increasing dark avoidance behavior, participates in a diurnal rhythm out of phase with norepinephrine [9]. Serotonin levels are high during daylight while norepinephrine levels rise at night. The elevated norepinephrine acts, via cyclic AMP, to induce the biosynthesis of the enzymes [3,11] forming melatonin, causing its nocturnal increase [14].

In spite of the circadian complexity of the pineal gland, we have produced pineal-dependent increases in dark avoidance in goldfish by treating them in ways designed to elevate their pineal serotonin or N-acetylserotonin. This was accomplished by direct injection of these compounds or by the injection of S-adenosyl-homocysteine which inhibits HIOMT.

Although N-acetylserotonin was more potent than serotonin in increasing dark avoidance behavior in goldfish, positive identification of which of these two is the primary effector awaits experiments performed with N-acetyltransferase inhibitors. However, it is predicted that administration of tryptophan, 5-hydroxytryptophan, harmine [8] or 6-methoxytetrahydro-β-carboline [13] would elevate the levels of both serotonin and N-acetylserotonin to increase dark avoidance in goldfish.

Since a major goal of this work was to clarify how scotophobin increased goldfish dark avoidance, we chose as a working hypothesis that this behavioral peptide somehow elevated pineal NAS levels. The results of the subsequent experiments evaluating the effect of scotophobin upon pineal melatonin levels indicated that scotophobin both reduced the amount of melatonin in the pineal glands and blocked the increase in pineal melatonin that normally occurred when NAS was injected. Thus, it appeared that scotophobin indeed was able to elevate pineal NAS, and apparently did so by reducing its conversion to melatonin by HIOMT. That scotophobin could effectively reduce HIOMT activity was confirmed by the discovery that scotophobin potently inhibited purified bovine HIOMT The mechanism of the scotophobin inhibitor of HIOMT has been determined and will be the subject of a later report (Satake and Morton, to be published)

Thus, as a simplified overview, it appears that animals, trained by electric shock to avoid the dark, produce a peptide which can cause the elevation of pineal NAS Elevation of NAS (or serotonin) can bring about dark avoidance behavior, provided the pineal gland is present. The means whereby pineal NAS elevation influences behavior, and the applicability of these observations to learning in general must await the results of future studies

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