

AN ABSTRACT OF THE THESIS OF

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Title: HYDROXYINDOLE-O-METHYLTRANSFERASE (HIOMT)

ACTIVITY IN THE PINEAL OF CHINOOK SALMON

(ONCORHYNCHUS TSHAWYTSCHA)

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This study investigated the activity of hydroxyindole-O-methyltransferase (HIOMT) in chinook salmon (Oncorhynchus tshawytscha). Various body tissues, as well as several morphological brain divisions were tested for the presence of HIOMT. Activity was found in the retina, the dorsal portion of the diencephalon, and in the pineal gland of chinook. However, the activity was significantly higher in the pineal gland than in the other tissues. Kinetic properties and the effects of light on enzyme activity were examined for HIOMT from the pineal. Optimum reaction conditions were obtained when pineals were homogenized in

0.2 M sodium phosphate buffer, pH 7.6-7.8, incubated at a temperature of between 20° and 30° C, and reaction mixture concentrations of N-acetylserotonin (NAS) and S-adenosylmethionine (SAM) of 5.0×10^{-6} M and 1.1×10^{-5} M respectively. Apparent K_m values were found to be 2.5×10^{-7} M for NAS and 1.43×10^{-5} M for SAM. Evidence was found that suggests a positive relationship between fish length and pineal HIOMT activity. This prompted sorting fish into a narrow size range prior to investigating the effects of light on the enzyme. Specific activity of HIOMT was found to exhibit diurnal cycling when fish were maintained in a daily photoperiod of 12 hours of light and 12 hours of darkness. Specific activity continued to cycle in continuous darkness but did not cycle in continuous light or when light was given to the fish during the expected dark-time. It was found that the cycle in specific activity was probably an artifact in this system, since pineal protein content was shown to cycle diurnally 180° out of phase with the cycle in specific activity while HIOMT activity per pineal did not show any apparent cycling. Preliminary findings indicate that, although no diurnal cycling exists, HIOMT may undergo seasonal variation.

Hydroxyindole-O-methyltransferase
(HIOMT) Activity in the Pineal of
Chinook Salmon (Oncorhynchus tshawytscha)

by

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HYDROXYINDOLE-O-METHYLTRANSFERASE (HIOMT) ACTIVITY
IN THE PINEAL OF CHINOOK SALMON (Oncorhynchus tshawytscha)

I. BACKGROUND

The function of the pineal gland has been shrouded in mystery and speculation since it was first identified by the ancient Greeks. Greek philosophers recognized the unique anatomical location of the pineal in the human brain, but their studies into the function of the structure were confined mainly to discussion and not physical investigation. These discussions led to several inventive ideas as to the function of the pineal. One was that it served as the seat of the soul or possibly as a valve controlling the flow of thoughts in the brain (Axelrod, 1970). The first medical observations which suggested a possible function for the pineal were made in 1898, when Hubner described a condition in which the pineal of a six year old boy was destroyed by a tumor. The boy showed precocious sexual maturation which seemed to be related to the pineal tumor (Axelrod, 1970). A number of studies were conducted attempting to establish more concrete relationships between the pineal gland and sexual maturation. Many of the early experiments were reviewed by Kitay and Altschule (1954). This area of investigation still remains one of very active research (see Reiter, 1973, for review).

Experiments of a more biochemical nature showed that bovine pineal extracts, when fed to tadpoles and frogs, caused rapid blanching of their skin (McCord and Allen, 1917; Hogben and Slome, 1931). Attempts to isolate the factor responsible for this blanching effect proved to be fruitless, until Lerner et al. (1958) were able to isolate the compound from bovine pineals. Characterization of this compound revealed it to be an indole, N-acetyl-5-methoxy-tryptamine, given the common name melatonin (Lerner et al., 1958, 1959).

Studies involving the physiological function of melatonin have taken several interesting routes. One of these has been the effects melatonin has on body coloration. Although these experiments actually began with McCord and Allen (1917), it was not until the identification of melatonin that investigators knew what compound they were dealing with. Bagnara (1963) demonstrated that melatonin dissolved in tap water was able to cause the aggregation of pigment granules in larval Xenopus melanophores. Hafeez (1970) was able to show significant skin blanching in rainbow trout (Salmo gairdneri), both by injecting melatonin intraperitoneally and by dissolving it in the aquarium water. When melatonin was added to the water, the night-time pigment spots of the pencil fish (Nanostomus beckfordi anomalus) became pronounced while the day-time

pattern faded (Reed, 1968; Ruffin et al., 1969). In a similar manner, trout maintained with a black background underwent nocturnal blanching (Hafeez and Quay, 1970a). The nocturnal blanching was still apparent when blinded fish were used, but the effect was absent if the blinded fish were also pinealectomized. This, coupled with the evidence that the pineal is the major source of melatonin, led to the hypothesis that increased pineal melatonin production and/or release at night caused the nocturnal blanching effect. Contrary to these findings, Owens et al. (1978) reported that despite a consistent nocturnal rise in plasma melatonin levels, adult rainbow trout did not undergo nocturnal blanching, regardless of background color. Studies involving the effects of melatonin on mammals show that although slower hair growth is seen in melatonin treated animals, no pigment changes are noticed (Houssay et al., 1966; Snell, 1965). Considering all the evidence, melatonin appears to have an effect on pigmentation of juveniles of many lower vertebrates, but has no noticeable effects on pigmentation of mammals or the adults of lower vertebrates.

Another proposed physiological function of melatonin, which may be related to changes in pigmentation discussed above, is the control of production and/or release of pituitary hormones. Panda and Turner (1968) found that

plasma TSH levels were significantly lowered in rats injected subcutaneously with 100 ug melatonin when compared to those receiving only a saline injection. Kamberi et al. (1970, 1971) reported that injection of melatonin into the third ventricle of the brain of rats stimulated prolactin release but inhibited LH and FSH release from the pituitary as measured by plasma levels of the hormones. However, melatonin perfused into the anterior pituitary by way of a cannulated hypophyseal portal vessel resulted in no changes in plasma LH, FSH, or prolactin. Adams et al. (1965) found that subcutaneous injection of melatonin increased the pituitary LH content. This was interpreted to mean that there was a decreased LH release while LH synthesis in the pituitary continued. As concluded by Reiter (1973), these data appear to show that melatonin has the capability to inhibit the hypothalamic releasing factors of LH and FSH, and also inhibit the release inhibiting factor for prolactin. Melatonin has also been shown to have an effect on somatotropin (Smythe and Lazarus, 1974a, 1974b) and MSH (Kastin and Schally, 1967; Kastin et al., 1967). While the experiments on the effect of melatonin on somatotropin and MSH are inconclusive, they do support the contention that melatonin is an important regulatory factor in the production and/or release of pituitary hormones.

In view of the suspected importance of melatonin, a

number of researchers began soon after the isolation of melatonin to investigate the biosynthetic and metabolic pathways of the compound (see Wurtman and Axelrod, 1968, for review). The synthesis of melatonin from the amino acid tryptophan is presented diagrammatically in Figure 1 (from Axelrod, 1970). While the entire sequence has been studied in only a limited number of species (Binkley et al., 1973; Wurtman and Axelrod, 1968; Wurtman et al., 1968), it is believed that this represents the scheme found in all vertebrates capable of synthesizing melatonin.

At least in rats, the final enzyme in the biosynthetic pathway of melatonin, hydroxyindole-O-methyltransferase (HIOMT) (E.C. 2.1.1.4.), has been found to have the lowest activity (Wurtman et al., 1963; Lovenberg et al., 1967). HIOMT has also been found to be highly localized in the pineal of mammals (Axelrod et al., 1961) and in the pineal and retina of lower vertebrates (Quay, 1965; Axelrod et al., 1965). For these reasons, it has been suggested that melatonin synthesis takes place primarily in the pineal (pineal and retina of lower vertebrates) and that the synthetic rate is controlled by HIOMT (Wurtman and Axelrod, 1968; Wurtman, Axelrod, and Kelly, 1968).

Much of the data accumulated for the proposed physiological role of melatonin has pointed to processes which are cyclic in nature. These include both diurnal

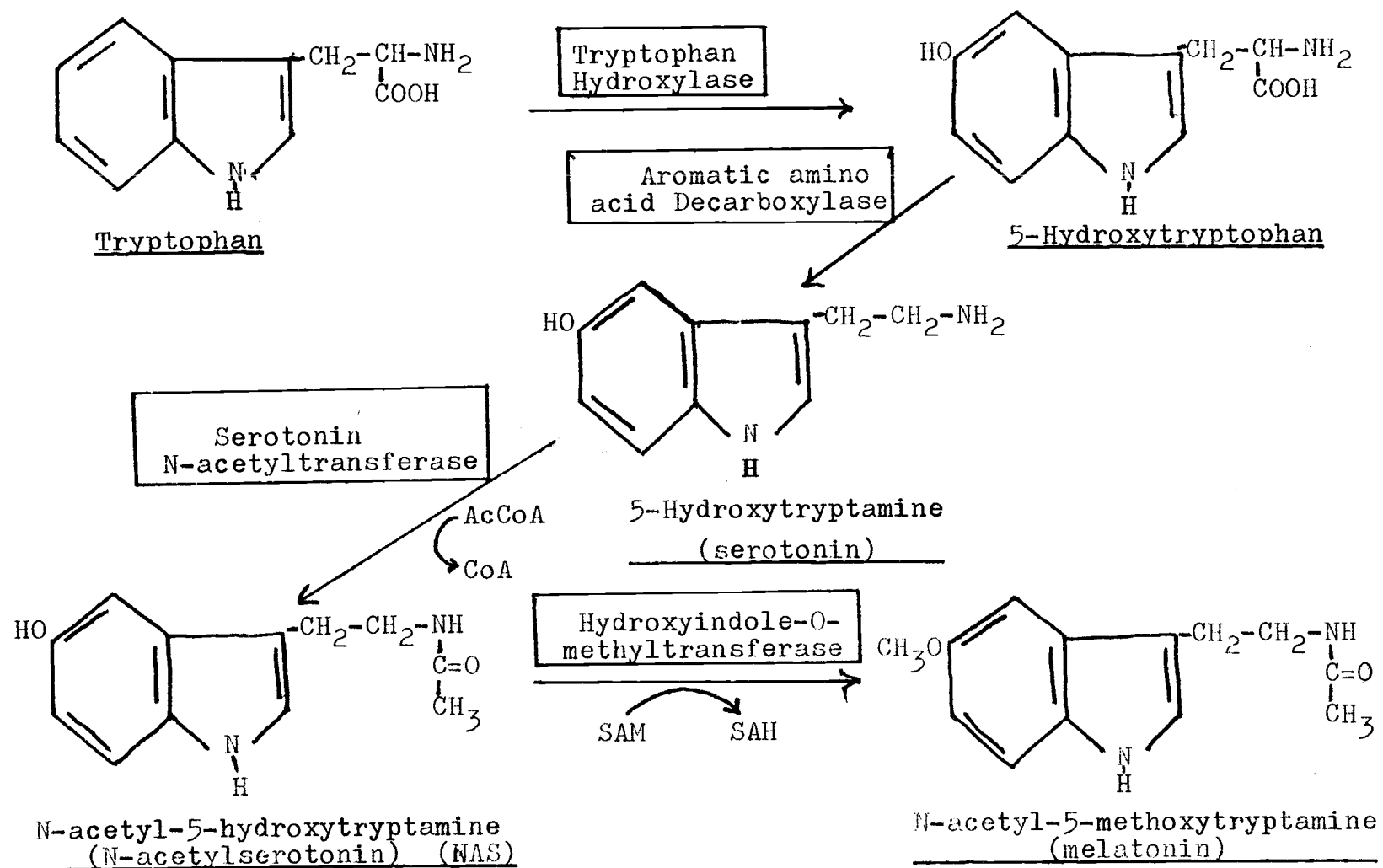


Figure 1. Biosynthesis of melatonin from tryptophan.

cycles, as in pigment changes, and seasonal cycles, as seen in reproductive rhythms and migration. Direct measurement of melatonin in body tissues was initially cumbersome and inaccurate. Because of this, investigators used HIOMT activity as a measure of melatonin forming potential, assuming that actual melatonin production would parallel HIOMT activity (Axelrod and Wurtman, 1968). Early studies on the diurnal behavior of HIOMT demonstrated that HIOMT activity underwent cycling that could be correlated with diurnal lighting conditions (Wurtman et al., 1963; Wurtman, Axelrod, and Kelly, 1968; Axelrod et al., 1965). To date, a number of different species have been examined and in most, diurnal cycling of HIOMT activity has been found (Wurtman, Axelrod and Kelly, 1968; Eichler and Moore, 1975; Smith and Weber, 1974). Recent advances in assay technology have made it possible to measure accurately melatonin in body tissues (Ralph et al., 1971; Rollag and Niswender, 1976; Wurzbberger et al., 1976). These studies have found that melatonin also undergoes diurnal cycling which corresponds to cycles seen earlier in HIOMT activity.

Not all studies conclude that there is a direct correlation between melatonin and HIOMT activity (Lynch and Ralph, 1970; Gern et al., 1978). In rats, the latest research shows little, if any, diurnal cycle in HIOMT while melatonin shows dramatic cycling (see Klein, 1978, for review). It was concluded that another enzyme in the

biosynthetic pathway leading to melatonin, N-acetyltransferase (see Figure 1), was responsible for the diurnal fluctuation seen in melatonin. These investigators speculated on the possible role of HIOMT as a seasonal rather than a daily regulator of melatonin synthesis. This question, however, has not yet been examined. In either case, diurnal or seasonal, HIOMT activity seems to be controlled by lighting conditions (Wurtman et al., 1963; Axelrod et al., 1965; Cardinali et al., 1971). This has led several investigators to propose that the major function of the pineal gland is to "transduce" lighting cues into a biochemical signal (ie. melatonin) and thus control or modify physiological processes (Axelrod, 1974; Klein, 1978).

The evidence presented for diurnal cycling of HIOMT activity comes primarily from mammalian and avian studies. Relatively few studies have examined this enzyme in detail from lower vertebrate sources. The few studies involving teleost HIOMT activity have led to conflicting reports, both as to the existence of diurnal HIOMT cycling and to the correlation between HIOMT activity and in vivo melatonin concentrations (Hafeez and Quay, 1970b; Smith and Weber, 1974, 1976b; Owens et al., 1978; Gern et al., 1978). These problems were the impetus for the studies presented here.

The intent of this thesis was to establish the existence of HIOMT in chinook salmon (Oncorhynchus tshawytscha),

to examine the reaction characteristics of the enzyme in detail, and to investigate HIOMT activity under diurnal lighting conditions. To facilitate presentation of the findings, the thesis is divided into two major sections: 1) Characterization of HIOMT; 2) Effects of light on HIOMT activity. Each of these sections is introduced separately in more detail.

II. CHARACTERIZATION OF HIOMT ACTIVITY

Introduction

Numerous species have been examined for the effects of photoperiod on hydroxyindole-O-methyltransferase (HIOMT) activity (Wurtman et al., 1963; Axelrod et al., 1964, 1965; Alexander et al., 1970a; Eichler and Moore, 1975; Smith and Weber, 1974). In several cases, though, failure to characterize thoroughly the reaction conditions has caused investigators to question the exact meaning of the results obtained (Weiss, 1968; Pelham and Ralph, 1972). Many researchers have merely adapted the assay conditions used by earlier workers, assuming that those conditions were applicable to their particular system.

Data from studies on a variety of animals seem to indicate that HIOMT from various pineal sources is somewhat different and that characterization of the enzyme activity for each species is required (Axelrod and Weissbach, 1961; Axelrod and Vesell, 1970; Alexander et al., 1970b; Eichler and Moore, 1975; Pelham and Ralph, 1972; Quay, 1971). Some characterization has been done on HIOMT from rainbow trout (Salmo gairdneri) (Smith and Weber, 1973; Smith, 1974) a closely related species to the chinook salmon used in this study. However, the possibility of species differences, noted above, prompted thorough examination of reaction parameters of chinook HIOMT activity

prior to investigating the effects of photoperiod on the enzyme activity.

Materials and Methods

Fish Culture

Spring chinook salmon (Oncorhynchus tshawytscha) from the Rogue River (Oregon) were reared from eggs at the Oregon Department of Fish and Wildlife Research Laboratory, Corvallis, Oregon. Fish were maintained outdoors in 1150 liter fiberglass tanks with running well water at $12.0^{\circ} \pm 1.0^{\circ} \text{ C}$, and were fed daily to repletion with Oregon Moist Pellets (Bioproducts). Juvenile fish, 15-20 cm, were used in these experiments.

Pineal Dissection

Fish were sacrificed by rapid decapitation. The cranial region was removed by a longitudinal cut through the mouth and above the orbit of the eye (see Diagram 1). The back portion of the skull was then removed by making a transverse cut across the optic lobes of the brain, which are visible externally as two dark circles. Under a dissecting scope (12X) the remaining optic lobe tissue was pulled away, exposing the pineal. Jeweler's forceps were used to grasp the pineal stalk, and pull out the pineal. Pineals from several fish were pooled and homogenized

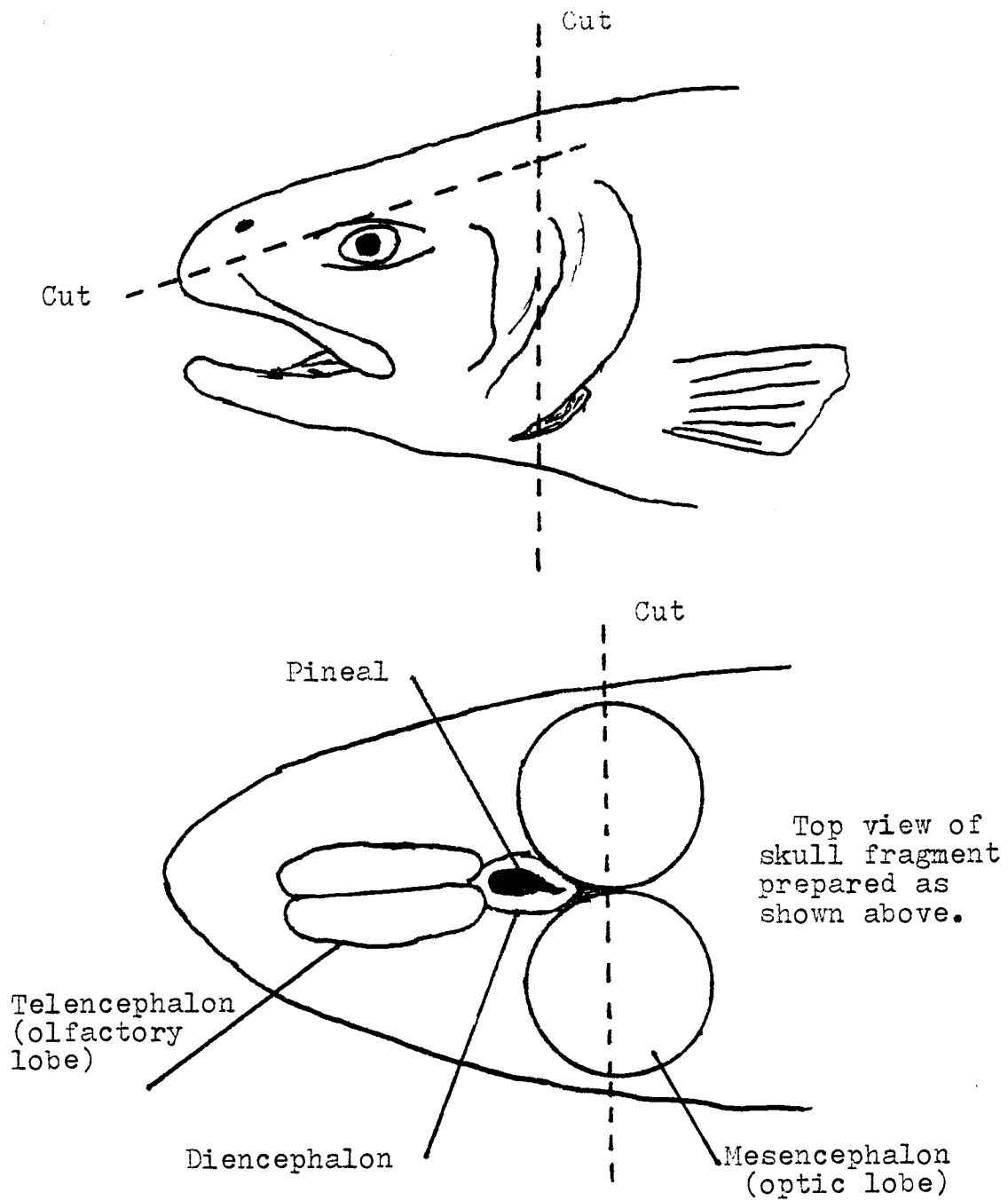


Diagram 1. Preparation of chinook skulls
for pineal dissection.

in ice cold 0.2 M sodium phosphate buffer, pH 7.6, to give a final homogenate concentration of approximately two pineals per milliliter. All homogenization was performed in a Kontes Potter-Elvehjem hand-operated glass-teflon tissue homogenizer.

HIOMT Assay

HIOMT activity was determined using a modification of procedures described by Hafeez and Quay (1970b), Smith (1976), and Smith and Weber (1974). The assay is performed in vitro and relies upon the formation of [^3H]-melatonin from N-acetylserotonin with [^3H]-methyl-S-adenosyl-methionine as methyl donor (see Figure 1). The [^3H]-melatonin is selectively extracted from the reaction mixture allowing determination of the amount of melatonin produced.

Pineal homogenates were centrifuged for 30 minutes at 12,000 x g (4⁰ C) in a Sorvall RC-2 superspeed centrifuge (SS-34 rotor). The supernatant was decanted and used in the subsequent analyses. For each experimental point, five 0.1 ml aliquots of the supernatant were transferred to 13 X 100 mm disposable culture tubes in an ice bath. Three of the tubes (assay) then received 50 ul N-acetylserotonin (NAS) and 25 ul [^3H]-methyl-S-adenosyl-L-methionine ([^3H]-SAM). The remaining two tubes (controls) received 25 ul [^3H]-SAM and 50 ul glass distilled water in

place of the NAS. The final reaction volume was 0.175 ml with final NAS concentration of 5.0×10^{-6} M, final [^3H]-SAM concentration of 1.1×10^{-5} M, and approximately 0.2 pineal per tube.

Reaction was initiated by placing the tubes in a 25°C shaking water bath (American Optical). Following a 10 minute incubation, the reaction was stopped by placing the tubes in an ice bath and immediately adding 1.0 ml of an ice-cold 0.2 M boric acid-KCl-NaOH buffer, pH 10.0, to each tube. The mixtures were extracted by addition of 2.5 ml toluene:isoamyl alcohol (80:20) followed by two vortex mixings. The phases were then separated by centrifuging five minutes at $1,000 \times g$ in a Sorvall GLC-2 tabletop centrifuge. Two ml of the organic phase from each tube were transferred to a scintillation vial and 10 ml BBOT-toluene scintillation fluor were added to the vial. Each vial was counted to at least 1.5% counting error (Miller, 1972) on a Packard Tri-Carb Liquid Scintillation Spectrometer (model 3310). Quench was checked periodically with a cesium external standard and with [^3H]-toluene as an internal standard.

The average counts from the two control tubes were subtracted from the average counts of the three assay tubes to give the total counts attributed to a product of the reaction that was subsequently identified as melatonin. Periodic checks of counting efficiency and

specific activity of the $[^3\text{H}]$ -SAM allowed conversion of counts to quantity of melatonin. Extraction efficiency of melatonin by the toluene:isoamyl alcohol was determined using $[^3\text{H}]$ -melatonin. This allowed the determination of the total amount of melatonin produced during the reaction.

Reaction mixtures with boiled enzyme preparation and with distilled water in place of the enzyme gave results similar to the control tubes. It was, thus, determined that the counts extracted from the control tubes probably represented nonspecific tritium rather than any other tritiated reaction product being formed.

When required, homogenate protein concentration was determined using a modification of the method described by Peterson (1977). For most kinetic studies, however, the protein concentration in the enzyme preparation was too low to allow accurate measurement. In order to check the buffering capacity in the reaction mixtures, the pH was checked where indicated. Due to the extremely small reaction volumes, the only practical method for measuring the pH was with Hydrion paper. Short range paper with a distinct color change every half pH unit was used, which allowed estimation of pH to within a quarter of a pH unit.

Chemicals

N-acetylserotonin (NAS) (Sigma Chem. Co.) was found to be stable diluted in glass distilled water at the

concentrations needed for at least three months while refrigerated. [^3H]-methyl-S-adenosyl-L-methionine (SAM) (New England Nuclear) and nonlabeled SAM (Sigma Chem. Co.) (mixed 4 ug/ml in glass distilled water) stock solutions were kept frozen at -20°C . Each week a fresh working solution of [^3H]-SAM was prepared by mixing appropriate aliquots of the two stock SAM solutions to give a final specific activity of 27-47 Ci/mole. The working solution was stored at 4°C and discarded at the end of the week.

BBOT (2,5-bis-2-(5-tert-butylbenzoxazolyl)-thiophene) (Sigma Chem. Co.) was mixed 4 grams per liter of scintillation grade toluene (Mallinckrodt Chem. Co.) to give the BBOT-toluene scintillation fluor used throughout this study. [^3H]-melatonin and [^3H]-toluene used were obtained from New England Nuclear, while all buffers, Hydrion paper, and nonlabeled melatonin were purchased from Sigma Chem. Co.

Anatomical Localization

In an attempt to determine the distribution of HIOMT, various body tissues were analyzed for enzymatic activity. Heart, liver, kidney, spleen, eye, epaxial musculature, as well as several morphological brain divisions were tested. Tissue samples were homogenized in 0.2 M sodium phosphate buffer, pH 7.6. HIOMT analysis and protein determinations were performed on the 12,000 x g supernatant as described above.

Results

Identification of the Reaction Product

Two separate methods were used to identify the reaction product that was extracted from the assay tubes. Ascending paper chromatography was performed using modifications of several systems described by other workers (Lerner et al., 1958; Axelrod and Weissbach, 1960; Klein and Notides, 1969). In all systems, samples from control and assay tubes were treated identically. Two ml of the toluene:isoamyl alcohol extract were concentrated to 0.1 ml under a stream of nitrogen. Fifty μ l of this concentrate were applied to Whatman #1 chromatography paper under nitrogen. Approximately 10 μ g melatonin dissolved in toluene:isoamyl alcohol (80:20) were spotted directly on top of each extraction sample. Following thorough drying under nitrogen, the chromatograms were then developed. Single dimensional chromatograms were developed in one of the following three systems: 1) isopropanol:ammonia:water (8:2:2); 2) n-butanol:acetic acid:water (120:30:50); 3) chloroform:methanol (94:6). Two dimensional chromatograms were developed using n-butanol;pyridine:water (1:1:1) followed by n-butanol:acetic acid:water (120:30:50). Visualization of melatonin was accomplished by short wavelength UV lamp. The chromatograms were then cut into sections, placed in scintillation vials with 10 ml BBOT-toluene fluor, and counted as described for the HICMT assay.

At 25° C, extraction of radioactive material into the toluene:isoamyl alcohol phase was linear with time (Figure 2). Paper chromatography of these extractions showed that approximately 90% of the total counts recovered co-chromatographed with melatonin. The remaining counts were divided between the origin, solvent front(s), and the rest of the paper. Chromatography of extractions from control tubes revealed no counts associated with melatonin. The majority of the counts ran with the solvent front(s) or remained at the origin.

The second method used to identify the tritiated compound produced during the reaction was recrystallization from benzene. Ten mg melatonin plus 5 uCi commercial [^3H]-melatonin were dissolved in 2.0 ml toluene:isoamyl alcohol (80:20). The solution was then evaporated to dryness under nitrogen, and the resulting residue dissolved in 2.0 ml hot benzene (75° C). Nondissolved material was filtered out by passing the hot solution through Whatman #1 filter paper. Melatonin was then allowed to crystallize, first at room temperature, then in an ice bath. Aliquots of 0.5 ml of the benzene solution, before and after crystallization, were counted in 10 ml BBOT-toluene fluor. The crystallized melatonin was then redissolved in hot benzene and the process repeated several times. Toluene:isoamyl alcohol extracts from both the control and assay tubes were taken through

the same procedure, except the commercial [^3H]-melatonin was omitted.

During the recrystallization procedure, 58% of the radioactivity, added as [^3H]-melatonin, crystallized from the benzene solution each time through the procedure. When toluene:isoamyl alcohol extracts were used, 57% of the radioactive material extracted from the assay tubes crystallized with melatonin, while none of the radioactivity from the extracts of control tubes crystallized with melatonin.

The results obtained from the paper chromatography and recrystallization led to the conclusion that the tritiated material produced during reaction in the assay tubes is indeed melatonin.

Stability of HIOMT Activity During Storage

Table 1 shows the stability of the enzyme preparation with various forms of storage. As can be seen, none of the simple storage methods tested help to extend the storing stability of the enzyme. Because of this, all studies were performed immediately following sample preparation.

Kinetic Characteristics

Production of melatonin was found to be linear with increased concentration of pineals in the reaction mixture (Figure 3). The use of approximately 0.2 pineal per

Figure 2. Melatonin production by HIOMT as a function of incubation time. Assay conditions were as described in Materials and Methods.

●————● 37° C, ■-----■ 30° C,
○-----○ 25° C, □————□ 0° C.

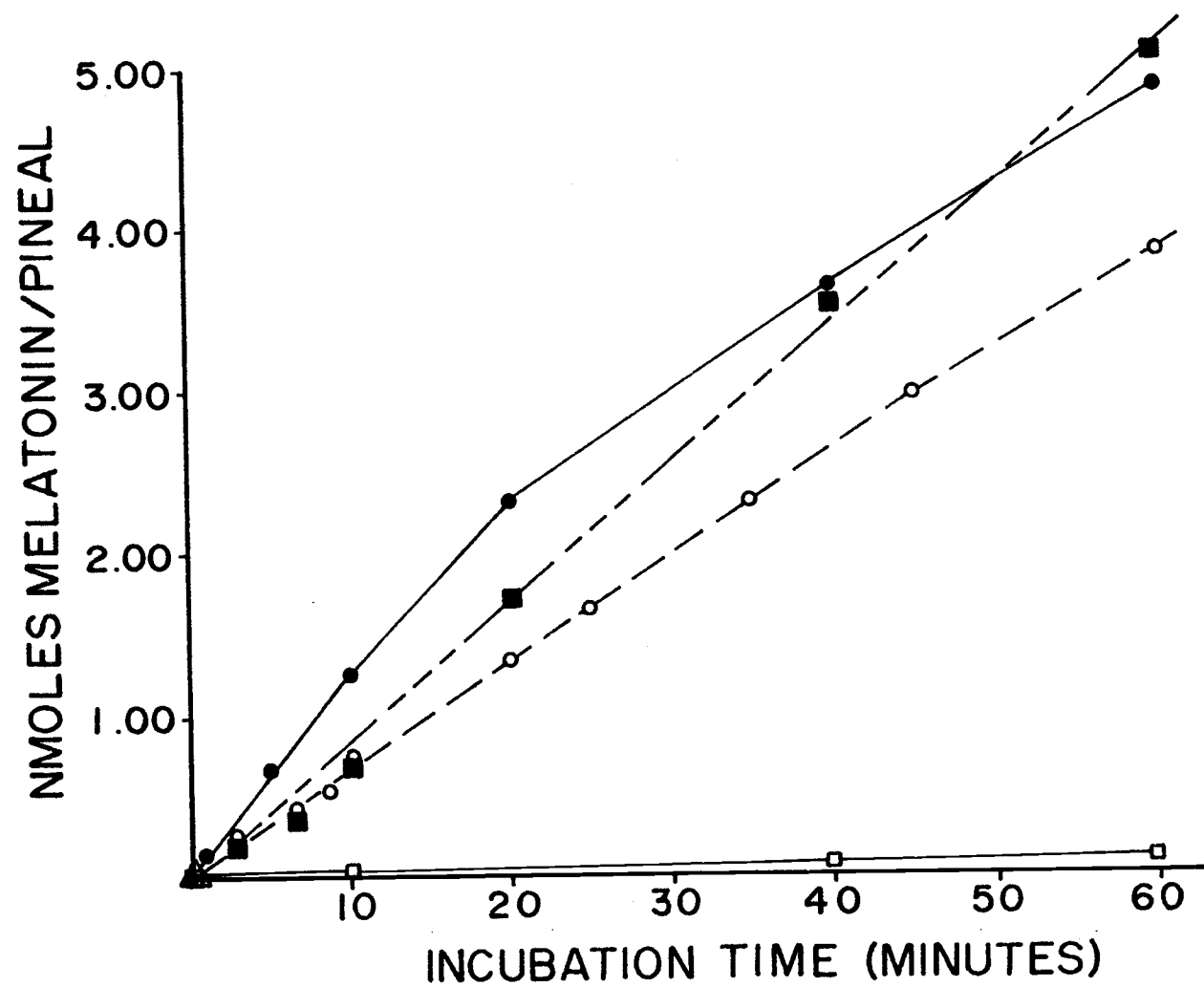
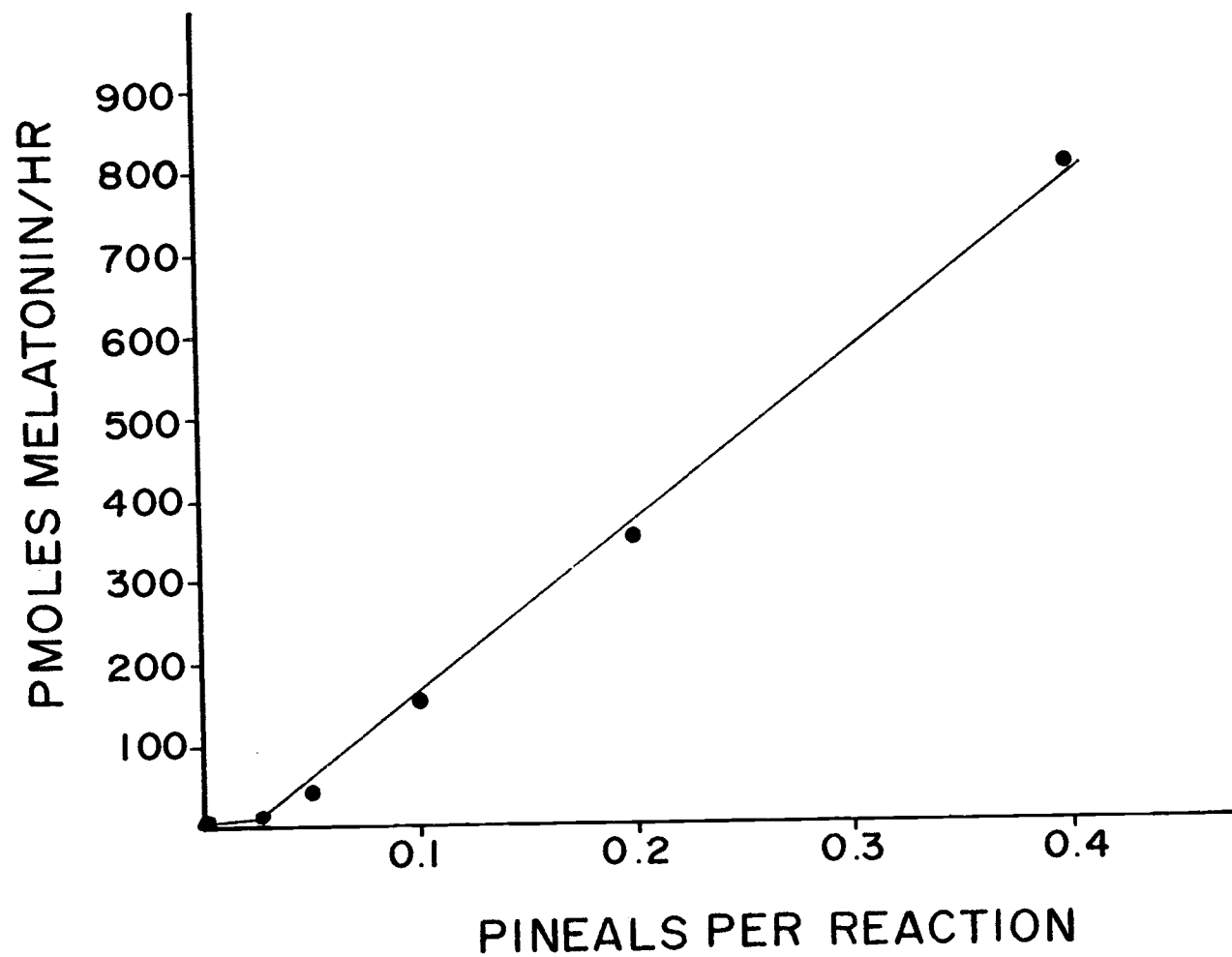


Table 1. Stability of HIOMT Activity During Storage. Conditions for the assay were as described for the general assay. Values indicate per cent activity remaining.**

Treatment	Hours Stored															
	0	1	2	4	6	10	12	16	24	48	96	120	168	192	400	480
Whole heads at 0°C \bar{x} of 5 at each pt.		100	74	68	65											
Skull fragments at -20°C \bar{x} of 5 at each pt.	100						86		66						41	
Pooled homogenate at 0°C \bar{x} of 3 at each pt. (2 pineals/ml)	100	100	110	98	98	89		84	84	73						
Pooled homogenate at -20°C \bar{x} of 3 at each pt. (4 pineals/ml)	100								94	52	22			2		0
(2 pineals/ml)	100								66	26	25			9		0
(2 pineals/ml + 2mM mercapto- ethanol)	100								0	0	0					0
(1 pineal/ml)	100								49	39	16	11		0		0

** Blank spaces indicate assay not performed.

Figure 3. Effect of pineal concentration on HIOMT activity.
Various pineal concentrations were obtained by diluting a concentrated preparation with the homogenizing buffer prior to adding to the reaction tubes. Other conditions were the same as described in the Materials and Methods.



reaction tube resulted in high HIOMT activity with the need for numerous pineals. For this reason, an approximate concentration of 0.2 pineal per reaction tube was used in all subsequent experiments.

The maximum reaction rate was obtained when the incubation temperature was 37° C (Figure 4). However, the enzyme system was not as stable at this temperature as it was at lower temperatures (Figure 2). Melatonin production was linear for only 20 minutes at 37°, while at 0°, 25°, and 30° C, the rate was linear for at least 60 minutes. To avoid stability problems, incubation was performed at 25° C in all subsequent studies.

The effect of pH on HIOMT activity was examined using a combination of 0.1 M Tris plus 0.1 M sodium phosphate as the buffer system. This gave good buffering capacity over the pH range used, as determined by measuring the pH of the reaction mixtures both before and after incubation. When a single buffer species was used, the addition of reactants altered the final pH, particularly at the extremes. As an example, 0.2 M sodium phosphate buffer was able to maintain the pH from 6.5 to 7.9. From 7.9 up, however, the addition of the reactants dropped the pH to below 8.0. For this reason, only the pH curves obtained using the Tris-phosphate combination are shown (Figure 5).

The pH optimum for HIOMT from pineals sampled in early January was 7.6 with a shoulder between 7.9 and 8.0.

When the experiments were repeated in late March, a slight shift in pH optimum to 7.8 was seen, while the shoulder shifted to between 8.0 and 8.5. Since characterization studies were initiated using the pH optimum obtained in January, pH 7.6 was used in all the experiments.

Results obtained when the buffering species was varied are shown in Table 2. Although imidazole gave better activity, sodium phosphate was chosen as the buffering species in subsequent experiments. Since sodium phosphate was the buffer used by most previous workers, it was chosen here to allow comparison with other studies. The possible existence of an imidazole methyltransferase (Axelrod and Vesell, 1970) which could be using imidazole as a substrate to form a tritiated product in the reaction mixtures also led to the selection of sodium phosphate instead of imidazole as the buffer for the reaction. Although further experiments have shown that chinook pineal preparations are not capable of utilizing imidazole as a substrate, the slight increase in activity that would be gained by using imidazole was not sufficient to warrant changing buffer species.

Optimum HIOMT activity was obtained at a sodium phosphate concentration of 30 mM (Figure 6). However, the variation in replicate assay tubes increased as the sodium phosphate concentration decreased (the coefficient of variation ($s/\bar{x} \cdot 100\%$) was 0.6% at 1003 mM sodium

Figure 4. Effect of temperature on HIOMT activity.
Assay conditions were the same as for the general HIOMT assay, except the incubation temperatures were varied as indicated.

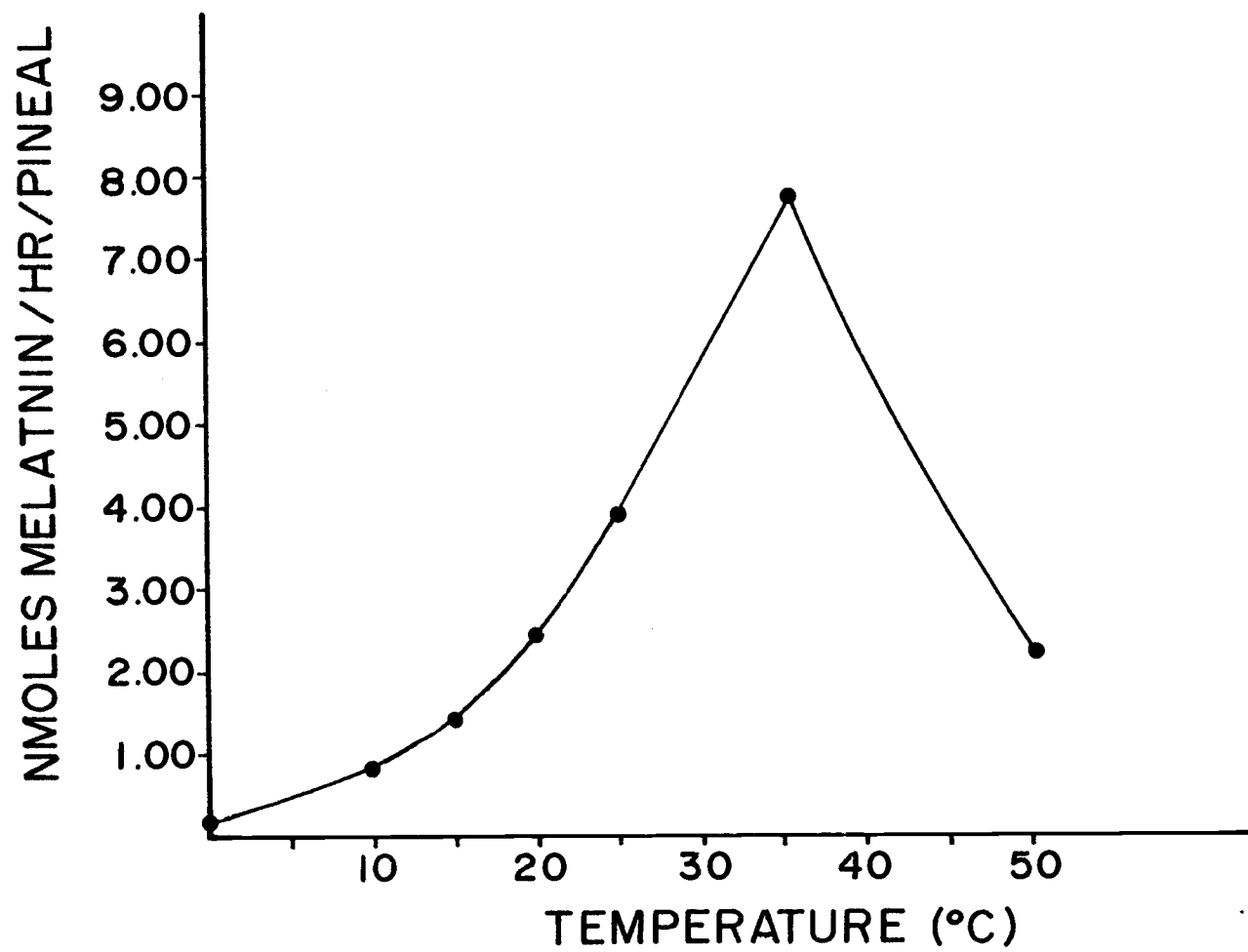


Figure 5. Effect of pH on HIOMT activity. Pineals were homogenized in 6 mM sodium phosphate buffer, pH 7.6, at 2X desired concentration. Reaction tubes received 50 ul pineal preparation and 50 ul buffer adjusted to give various pHs. All other conditions as outlined in Materials and Methods. ●——● determined in January, O-----O determined in March.

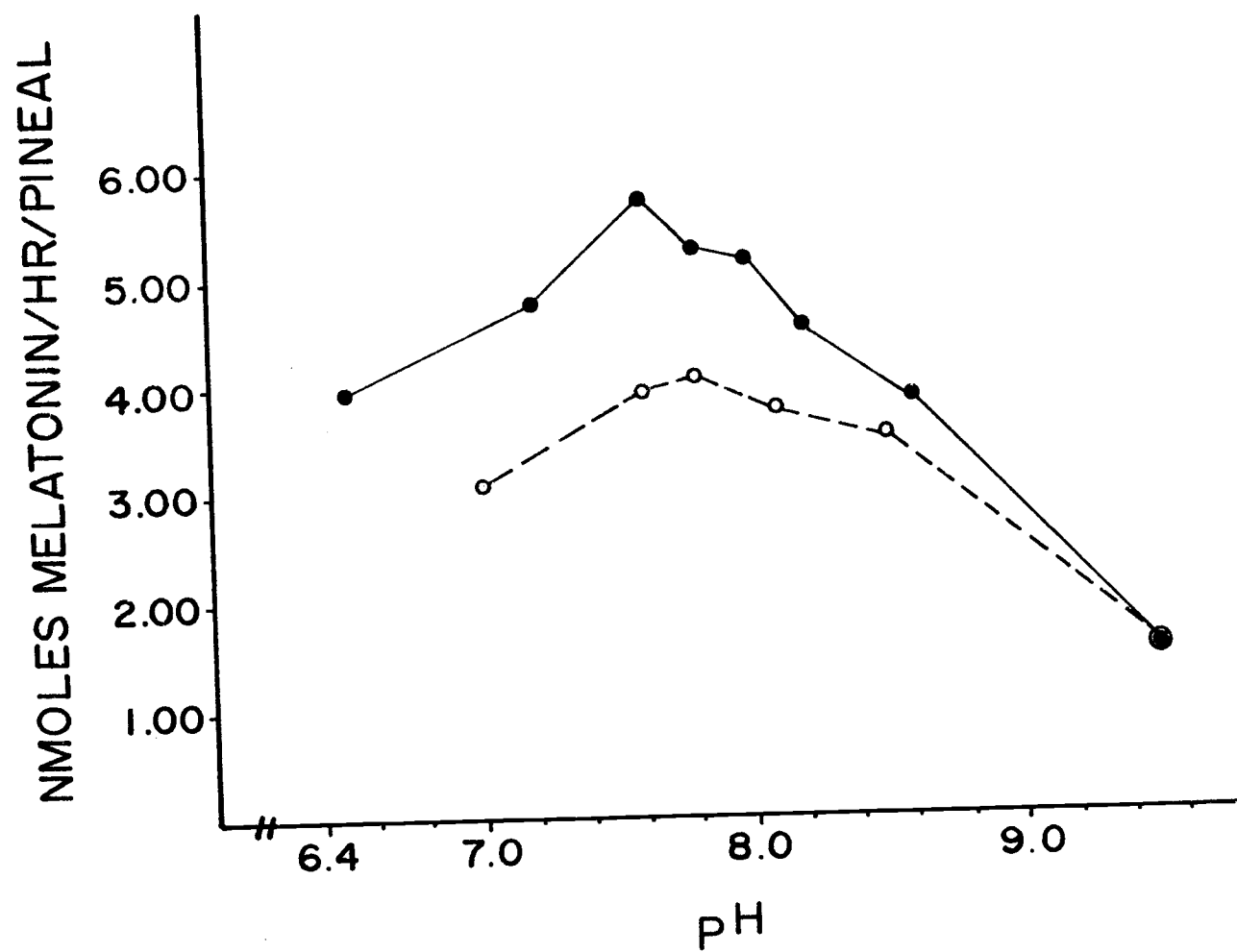
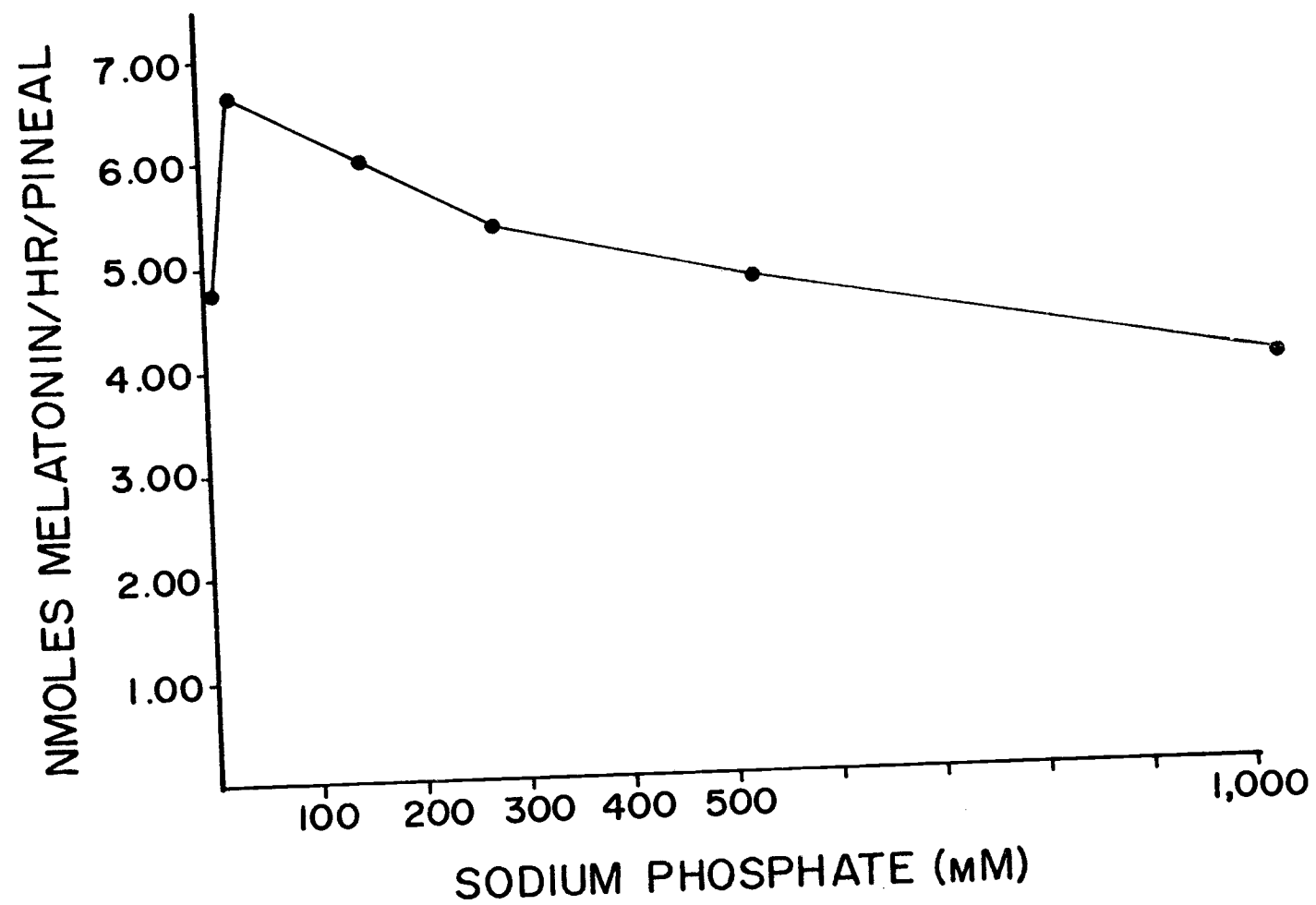


Table 2. Effects of buffer species on HIOMT activity.
 Pineals were initially homogenized in 6 mM sodium phosphate buffer, pH 7.6, at 4X the desired concentration. All other reaction conditions were as described in Materials and Methods, except that each tube received 25 ul of the 12,000 x g supernatant pineal preparation and 75 ul of the various buffer solutions, pH 7.6, to give final buffer concentration indicated.

Buffer Species	% Activity (Sodium Phosphate = 100%)
Tris (0.2 M)	36.4
Sodium Phosphate (0.2 M)	100.0
Tris + Sodium Phosphate (0.1 M + 0.1 M)	91.6
HEPES (0.2 M) (N-2-hydroxyethylpiperazine-N'- 2-ethanesulphonic acid)	90.4
Tricine (0.2 M) (N-tris hydroxymethyl methylglycine)	48.2
Imidazole (0.2 M)	118.8

Figure 6. Effect of sodium phosphate buffer concentration on HIOMT activity. Conditions were the same as given for Figure 5, except phosphate buffer concentration was adjusted, instead of pH, to give various buffer concentration.



phosphate, 10.1% at 30 mM, and 16.1% at 6 mM). Also, as the concentration increased, problems were encountered with the solubility of the sodium phosphate buffer in the ice bath prior to incubation of the reaction mixtures. For these reasons, 0.2 M sodium phosphate was chosen as the buffer in the homogenizing medium, which gave a final sodium phosphate concentration of 0.115 M.

Figure 7 shows the effect of NAS concentration on HIOMT activity. Optimum activity occurs at a concentration of 5.0×10^{-6} M, with an apparent K_m of 2.5×10^{-7} M NAS. All subsequent experiments were performed with the final NAS concentration at the optimum, 5.0×10^{-6} M.

The effect of varying the final SAM concentration on HIOMT activity is shown in Figure 8. A Lineweaver-Burk plot of these data (Figure 9) showed a V_{max} of 10.98 nmoles melatonin/hr/pineal with an apparent K_m of 1.43×10^{-5} M SAM. In all subsequent experiments a final SAM concentration of 1.1×10^{-5} M was used.

Subcellular Distribution of HIOMT Activity

Differential centrifugation of pineal homogenates revealed that the majority of the HIOMT activity is found in the 100,000 x g supernatant fraction (Table 3). No differences in reaction characteristics could be detected between crude homogenates and 12,000 x g supernatant fractions. For this reason, in the experiments on the

effects of light on HIOMT activity (see Section III) the 12,000 x g spin prior to HIOMT assay was eliminated.

Anatomical Localization

HIOMT activity is found not only in pineals, but also in the retina as well as the dorsal portion of the diencephalon of juvenile chinook salmon (Table 4). No other body tissue was found to contain any HIOMT activity. Although activity was found in the retina and dorsal diencephalon, specific activity (pmoles melatonin produced/hr/ug protein) in these tissues was considerable lower than that found in the pineal.

Seasonal Variation in HIOMT Activity

Table 5 shows HIOMT activity found in various months of this study. These values were obtained from particular experiments that were in progress during the designated month. While no attempt was made in the design of the experiments to allow accurate comparison of seasonal HIOMT activity, the differences seen do suggest the possibility of seasonal variation. Using a length vs activity relationship discussed in the next section (Figure 11), the predicted HIOMT activity of fish the size seen in January (maximum 20 cm) is 2.6 nmoles melatonin produced/hr/pineal. As such, the seasonal differences can

Figure 7. Effect of N-acetylserotonin (NAS) concentration on HIOMT activity. All conditions were as described in Materials and Methods, except NAS concentration in the final reaction mixture was varies as indicated. NAS stock was diluted prior to adding to the reaction mixture and delivered in a volume of 50 μ l.

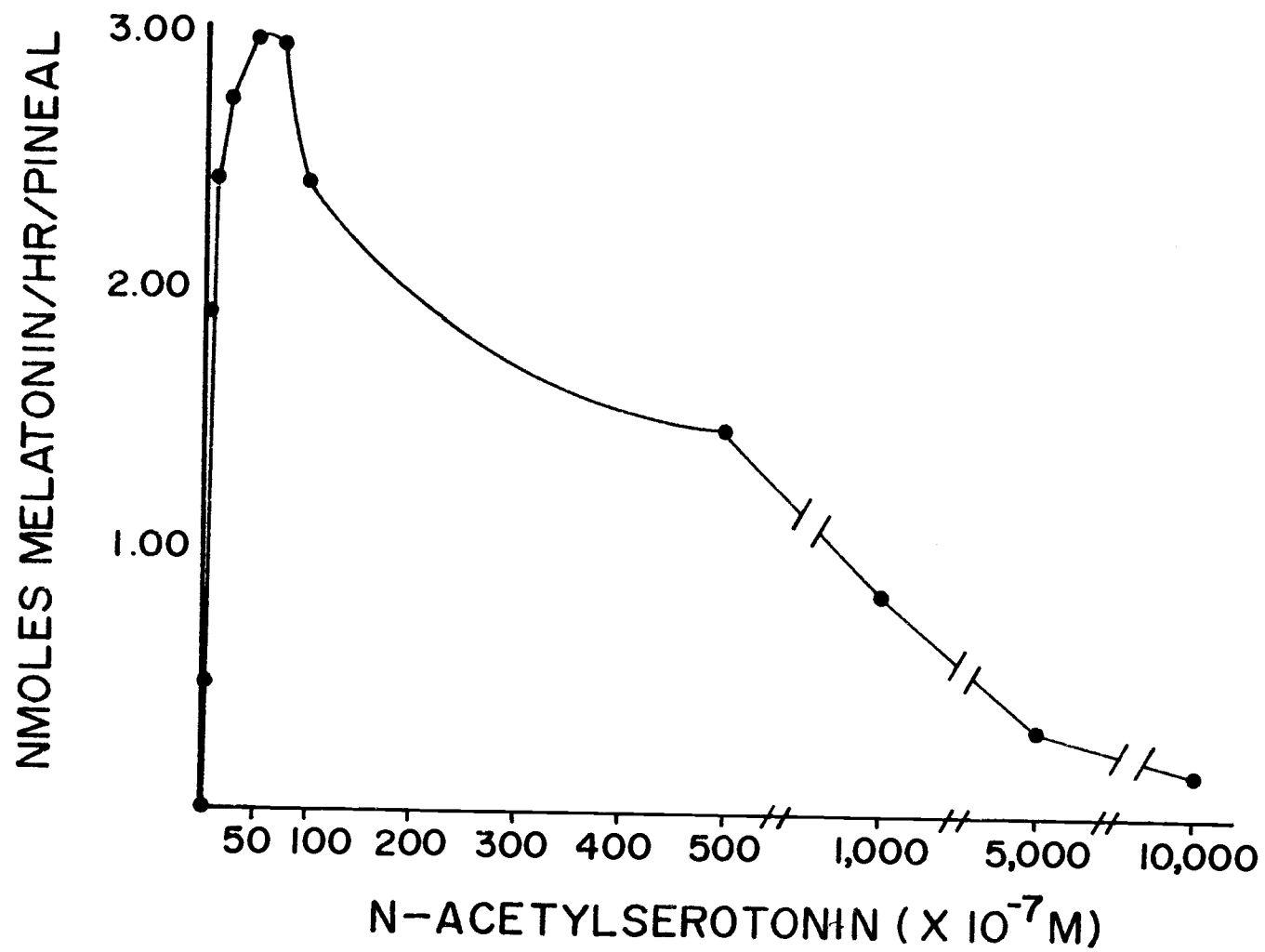


Figure 8. Effect of S-adenosylmethionine (SAM) concentration on HIOMT activity. SAM concentrations were varied by changing the amount of nonlabeled SAM while keeping the amount of [^3H]-SAM constant. A similar curve was produced when SAM concentrations were varied by dilution of concentrated solution of nonlabeled + [^3H]-SAM keeping the SAM specific activity the same throughout. All other conditions remained as for the general HIOMT assay.

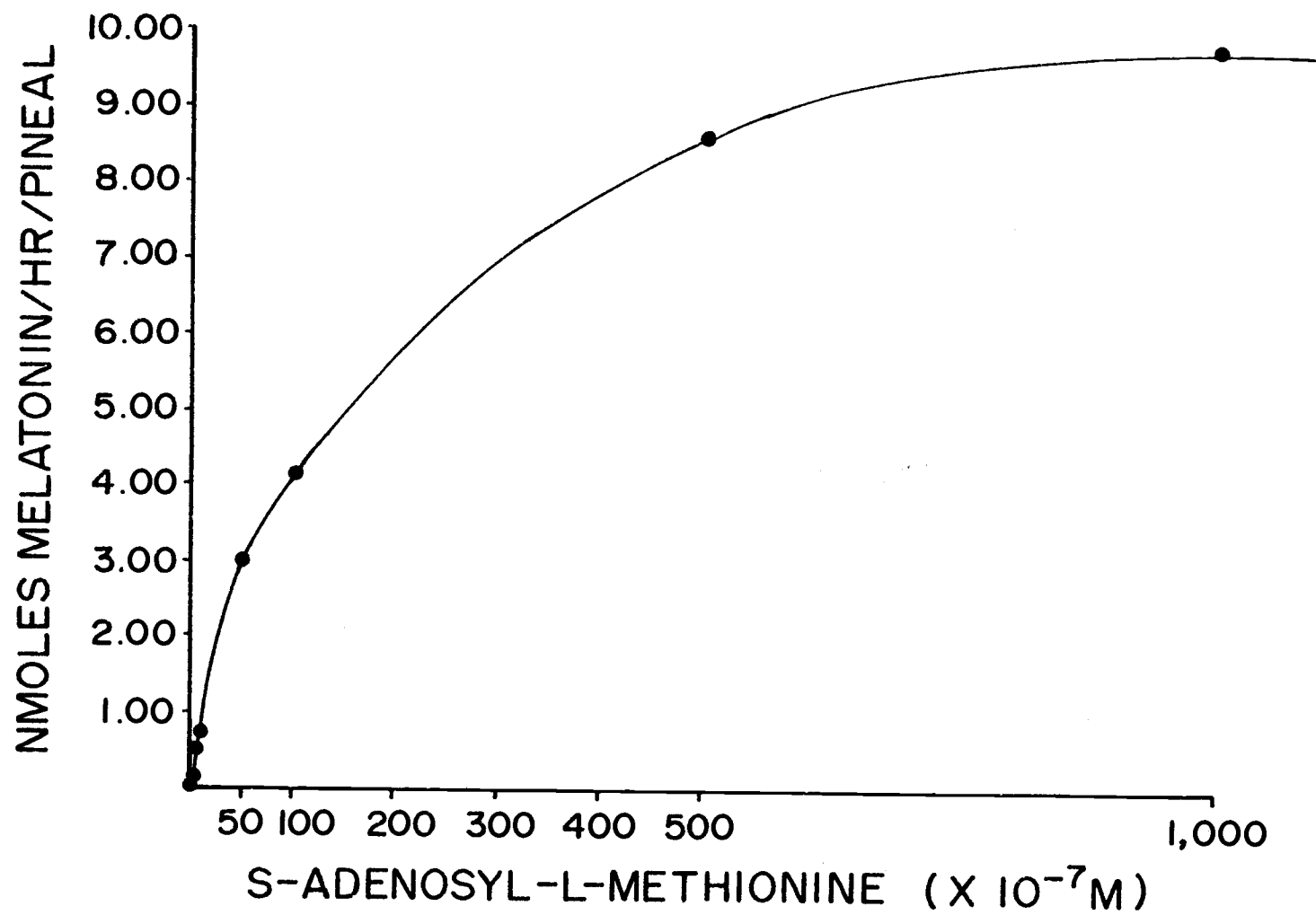


Figure 9. Lineweaver-Burk plot of the data from Figure 8.

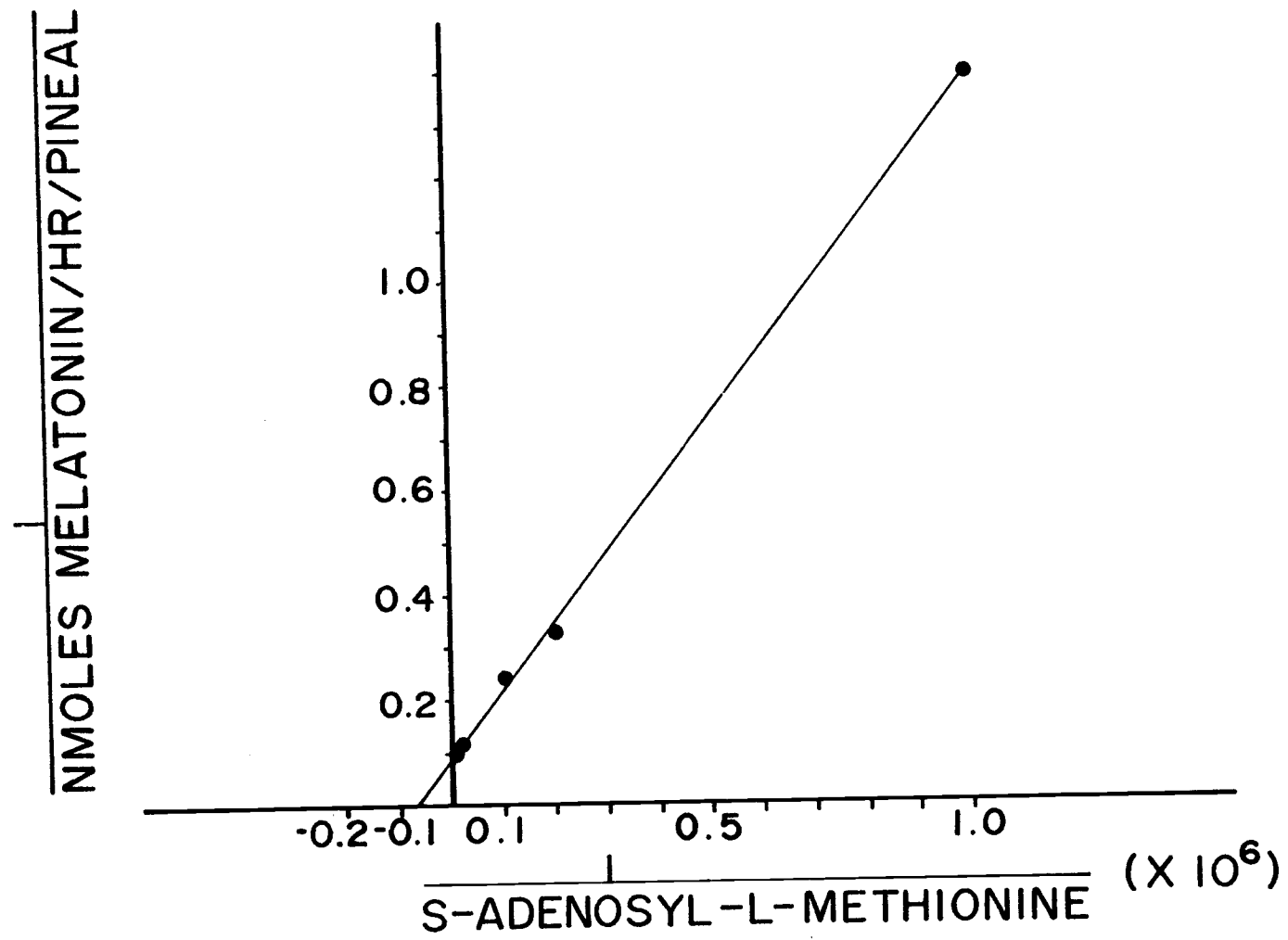


Table 3. Subcellular fractionation of HIOMT from chinook pineals.

Fraction	Protein		HIOMT Activity		Specific Activity
	ug	% Crude	pmoles/hr*	% Crude	pmoles/hr/ug protein**
Crude Homogenate	651	100.0	5584	100.0	8.58
1,000 x g pellet	136	20.9	126	2.3	0.93
10,000 x g pellet	84	12.9	109	2.0	1.30
30,000 x g pellet	27	4.1	50	0.9	1.85
100,000 x g pellet	13	2.0	24	0.4	1.85
100,000 x g supernatant	238	36.6	4875	87.3	20.48
% Recovery		76.5		92.9	

* pmoles melatonin produced/hour

** pmoles melatonin produced/hour/ug total pineal protein

Table 4. HIOMT activity in various body tissues of chinook salmon. All activities were determined as in the general assay. N.D. indicates activity was not detectable using this technique.

Body Tissue	Specific Activity ^a $\bar{x} \pm \text{S.D. (N)}$
Heart	N.D. (3)
Spleen	N.D. (3)
Liver	N.D. (3)
Kidney	N.D. (3)
Epaxial Musculature	N.D. (3)
Brain	
Telencephalon (olfactory lobe)	N.D. (7)
Diencephalon	
Dorsal	0.52 \pm 0.37 (7)
Ventral	N.D. (5)
Mesencephalon (optic lobe)	N.D. (7)
Eye	
Retina	3.10 \pm 1.39 (6)
Vascular layer	N.D. (5)
Pineal (August)	16.26 \pm 2.00 (20)

^a pmoles melatonin produced/Hr/ug pineal protein

Table 5. Seasonal HICMT Activity. Activities were determined as described for the general assay, with all values standardized to an incubation temperature of 30° C.

Month	Fork Length $\bar{x} \pm \text{S.D. (N)}$	HICMT Activity ^a $\bar{x} \pm \text{S.D. (N)}$
<u>1976 Brood</u>		
December	12.8 \pm 2.22 (30)	2610 ^b
January	13.6 \pm 2.67 (30)	4072 ^b
February	14.6 \pm 2.62 (30)	3765 ^b
March	16.9 \pm 2.10 (30)	2162 \pm 680 (5) ^c
<u>1977 Brood</u>		
May	8.2 \pm 0.47 (10)	267 \pm 24 (10) ^c
September	11.0 \pm 0.25 (10) ^d	1120 \pm 106 (10) ^c
October	11.0 \pm 0.25 (10) ^d	1291 \pm 310 (10) ^c

^apmoles melatonin produced/hr/pineal

^bTriplicate samples from pooled homogenates

^cPineals sampled individually

^dSize sorted prior to sampling

Table 6. Analysis of Variability. All values were determined as described in the text.

Sample	$\bar{x} \pm \text{S.D. (N)}$	Coefficient of Variation ^a
Multiple aliquots from a single pineal homogenate		
HIOMT assay tubes ^b	1183 \pm 46 (15)	3.9 %
HIOMT control tubes ^b	239 \pm 33 (15)	13.8 %
Pineal protein ^c	11.6 \pm 0.4 (10)	3.4 %
0.1 ml aliquots from individual pineal homogenates		
HIOMT activity ^d	114.12 \pm 12.55 (20)	11.0 %
HIOMT specific activity ^e	13.33 \pm 1.06 (20)	7.9 %
Pineal protein ^c	8.56 \pm 1.32 (20)	15.4 %

^a $s/\bar{x} \cdot 100\%$

^b cpm/aliquot

^c ug protein/aliquot

^d pmoles melatonin produced/hour

^e pmoles melatonin produced/hour/ug protein

not be entirely attributed to changes in fish size, and thus, involvement of factors other than growth are implied in seasonal variation of HIOMT activity seen in this study.

Analysis of Variability

Samples were analyzed to determine the variability that exists in the assay system and among individual pineals. Multiple aliquots from the same homogenate gave a coefficient of variation ($s/\bar{x} \cdot 100\%$) of 3.9% in radioactivity extracted from assay tubes (Table 6), while 13.8% variation was found in radioactivity from control tubes. Variation in replicate protein samples from the same homogenate was found to be 3.4%. When pineals were homogenized individually, 11.0% variation was found in HIOMT activity (pmoles melatonin/hr/pineal). Variation in the amount of protein per pineal was 15.4%, while variation in HIOMT specific activity (pmoles melatonin/hr/ μ g protein) among individual pineals was found to be 7.9%.

Discussion

The reaction characteristics of HIOMT activity from various vertebrate sources are compared in Table 7. HIOMT from chinook pineals is found to have properties that are similar in some respects but in others quite different from HIOMT found in other vertebrates (Axelrod and

Table 7. Comparison of kinetic properties and specific activities of HIOMT from different vertebrate sources. Values are those listed in the literature. No attempts have been made to standardize values.

Source	pH optimum	Temperature optimum (°C)	Apparent K_m		Specific Activity	Author
			SAM	NAS		
Cow pineal	7.5-8.3	37° ^a	$4.6 \times 10^{-5} M$	$5.4 \times 10^{-5} M$	2.96 ^d	Axelrod, 1961
Rat pineal	8.0 ^a	37° ^a	$1.1 \times 10^{-5} M$	Not Given	29 ^e	Weiss, 1968
Chicken pineal	7.9 ^a	37° ^a	$2.0 \times 10^{-4} M^b$	$2.0 \times 10^{-5} M^b$	135 ^f	Pelham, 1972
Frog Diencephalon Neural Retina	8.6 8.4	37° ^a	$7.1 \times 10^{-9} M$ $7.8 \times 10^{-9} M$	$5.3 \times 10^{-6} M$ $8.5 \times 10^{-6} M$	335.6 ^g 2678.8 ^g	Eichler and Moore, 1975
Fish pineal	7.6, 7.8	37° ^c	$1.4 \times 10^{-5} M$	$2.5 \times 10^{-7} M$ ($5.0 \times 10^{-6} M^b$)	Jan. 4072 ^h 472 ⁱ July 484 ^h 17 ⁱ	Present Study

^aused but not shown to be optimum

^boptimum concentrations

^cgave greatest activity but not used due to possible stability problems

^dug melatonin produced/hr/mg pineal protein

^epmoles melatonin produced/min/mg pineal protein

^fnmoles melatonin produced/hr/mg tissue

^gpM melatonin produced/hr/mg protein

^hpmoles melatonin produced/hr/pineal

ⁱpmoles melatonin produced/hr/ug pineal protein

Weissbach, 1961; Weiss, 1968; Pelham and Ralph, 1972; Eichler and Moore, 1975).

Optimum pH values reported for different species range from 7.5 to 8.6. Most investigators have used sodium phosphate for buffering. However, in this study 0.2 M sodium phosphate was unable to maintain the desired pH when the reactants were added if the initial buffer pH was above 7.9. To avoid problems of buffering capacity at pHs above 7.9, the pH values reported here were obtained using a combination of Tris and sodium phosphate. This combination was found to have acceptable buffering capacity over the pH range tested.

The differences in pH optimum of chinook HICMT between samples taken in January and March (Figure 5) are quite intriguing. The fact that HICMT from various vertebrate species exhibits differing kinetics suggests the existence of different forms of HICMT. Axelrod and Vesell (1970) demonstrated that HICMT from cows differs significantly in a number of factors from quail HICMT. Data presented by Eichler and Moore (1975) suggest that different forms of the enzyme may even exist in various tissues of the same individual. In more elaborate studies, Jackson and Lovenberg (1971) were able to show by column

chromatography that HIOMT from bovine pineals exists as two differently charged proteins which also may be capable of forming higher molecular weight aggregates. While they did not examine the kinetic characteristics of each form, their data suggest that differences in activity exist among the various forms. The changes in pH optima found in the present study hint at the possible existence of isozymic forms of HIOMT in chinook pineals, which undergo shifts in predominance at different times of the year. Since HIOMT activity was determined at a single pH (7.6) throughout the year, the shifts in pH optimum may be at least partially responsible for the seasonal difference seen in HIOMT activity in this study (Table 5).

HIOMT from various sources seems to show the highest activity at temperatures around 37° C. Chinook pineal HIOMT also shows the highest activity at this temperature, but linear melatonin production is only seen for a limited reaction time (Figure 2). This suggests that there may be stability problems with the enzyme at this temperature. Problems of this type were mentioned in work reported by Smith (1974) in rainbow trout, a species closely related to chinook salmon.

Reported K_m values for the substrates of HIOMT show considerable variation among the different sources tested. The utilization of SAM in the reaction, in all cases appears to follow the usual Michaelis-Menten kinetics.

Apparent K_m s for SAM range from 7.0×10^{-9} M to 4.6×10^{-5} M. The value obtained in this study, 1.43×10^{-5} M, is comparable to that found in mammalian studies.

Experiments investigating the use of NAS by HIOMT show conflicting results. In the cow (Axelrod and Weissbach, 1961) and frog (Eichler and Moore, 1975), the findings indicate Michaelis-Menten kinetics, however, in the chicken (Pelham and Ralph, 1972), quail (Alexander et al., 1970b), lizard (Quay, 1971), and in the present study, an optimum NAS concentration exists, with increased NAS concentrations actually inhibiting HIOMT activity (Figure 7). Careful examination of possible substrate regulation of HIOMT activity, as well as discrepancies found among species for the effect of NAS on HIOMT will need to be undertaken in order to answer the many questions remaining.

In all studies, including the present one, where subcellular localization was performed, HIOMT activity remains in the soluble fraction following centrifugation. This suggests that the enzyme is not bound to membranes but exists free in the cytoplasm. Anatomical localization of HIOMT also shows similarities among species. It has been found in the pineal and retina of most species studied, with only a few studies showing existence in other areas. Quay (1965) presented a survey of HIOMT activity in the retina and pineal of a number of vertebrates. He also suggested ubiquitous localization

in tissues of the Pacific lamprey (Hafeez and Quay, 1970b). Contrary to earlier reports, Cardinali and Rosner (1971) have even found the enzyme in mammalian retinas. The present study found HIOMT activity in the pineal, retina, and dorsal diencephalon (see Table 4), although activities in the retina and diencephalon were significantly lower than that of the pineal. It is also possible that the activity found in the dorsal diencephalon merely represents contamination from HIOMT in the pineal stalk, since it was impossible to ensure complete stalk removal when the pineal was taken.

Data presented in Table 5 suggest that seasonal variation may exist in HIOMT activity in the pineal of chinook salmon. One possible explanation for this variation is suggested by the shift in the pH optimum of the reaction seen between January and March (Figure 5). The studies of Jackson and Lovenberg (1971) predict at least two differently charged species of HIOMT in rat pineals. If a similar system exists in chinook with each species exhibiting a different pH optimum, a seasonal change in the concentration ratio of the species would appear in this assay system as seasonal variation in HIOMT activity. Since a single buffer pH (7.6) was used throughout the year in this study, the activity measured in the HIOMT assay would represent cellular activity only if the cellular pH remained the same throughout the year. However,

if the proposed change in the dominant species merely reflects seasonal changes in cellular pH, the variation seen seasonally in this study would represent an artifact of the assay.

Klein (1978) proposed another possible mechanism for seasonal variation in HIOMT activity. He speculates that variation in day length may act through a system in which light/dark can affect specific protein synthesis, and thus, affect HIOMT activity. It is suggested that HIOMT activity increases during darkness and decreases in light, but that the system is slow to respond to changes in lighting condition. Thus, in any single 24-hour light/dark cycle, only slight changes in activity can be detected. However, with increasingly longer dark periods (shorter days) more time would be available each night for increasing activity so that over a period of weeks the activity would gradually be seen to increase. The converse of this would be expected to happen as day lengths become longer (shorter nights). There would be more time for the activity to decrease during the day and less for increasing activity at night, so the overall activity would gradually decrease. This type of relationship has been seen in rats that were subjected to continuous light or continuous darkness for a period of several weeks (Axelrod et al., 1965b). HIOMT activity was decreased in continuous light and increased in continuous darkness

when compared to activity in rats receiving diurnal lighting.

The explanations offered for seasonal variation in HIOMT activity will have to be examined in more detail before the mechanism for the seasonal changes seen in this study can be determined. While it is suggested here that the variation seen may be merely an artifact, the suspected physiological function of melatonin fits quite neatly into a system in which seasonal cycles actually exist, and thus, provide a mechanism for "transducing" seasonal photoperiod cues into a physiological cue in the form of melatonin.

III. EFFECTS OF LIGHT ON HIOMT ACTIVITY

Introduction

Since the discovery that lighting conditions can affect HIOMT activity (Wurtman et al., 1963; Axelrod et al., 1964), many species have been examined for similar actions (Alexander et al., 1970b; Axelrod et al., 1965b; Binkely and Geller, 1975; Eichler and Moore, 1971, 1975; Hafeez and Quay, 1970b; Smith and Weber, 1974; and many others). In the majority of cases, a diurnal cycle in HIOMT specific activity (amount of melatonin produced per mg or ug protein) has been noted, with a peak occurring during the dark portion of the lighting cycle and a nadir occurring during the light portion. Studies involving rats have led to the conclusion that this cycle is controlled through a complex series of events in which light perceived by the eyes leads to an inhibition of a system that is responsible for a nocturnal change in protein synthesis, and thus an increase in HIOMT activity (Axelrod, 1974; Klein, 1978). An endogenous rhythm also appears to be present that allows the cycle to continue in the absense of light. Studies on steelhead trout (Salmo gairdneri) (Smith and Weber, 1974; Smith, 1976) have shown a diurnal cycle in HIOMT activity that is controlled entirely by light:dark conditions perceived through the eyes, with no apparent endogenous system. Hafeez and Quay

(1970b) found that continuous light or darkness had no effect on HIOMT activity in rainbow trout (a non-migratory form of Salmo gairdneri). The apparent discrepancies seen in the effects of light on HIOMT activity prompted this portion of the study. Experiments presented here examine possible photoperiod induced and/or endogenous rhythms of HIOMT activity in the pineals of chinook salmon.

Materials and Methods

Fish Culture

Juvenile Tule stock fall chinook salmon (Columbia River) were reared from eggs at the Oregon Department of Fish and Wildlife Research Laboratory in Corvallis, Oregon. Fish were maintained in running well water, $12.0^{\circ} \pm 1.0^{\circ} \text{C}$, indoors in 1150 liter fiberglass tanks under natural lighting conditions, and were fed once daily to repletion with Oregon Moist Pellets (Bioproducts).

Six days prior to the beginning of scheduled experiments, the necessary number of fish, 7 to 13 cm in length, were transferred to experimental tanks (96 liters) where photoperiod and exposure to extraneous light were precisely controlled (see Diagram 2 for arrangement of experimental room). Daily feeding was continued throughout the course of the experiments. Size sorting of fish

was found to be necessary following several experiments. Fish were sorted by size class by laying individual fish in a water-filled trough that was graduated for the size of fish desired. Only those fish which were within a 0.5 cm range were used in experiments requiring size sorting.

Pineal Dissection

At each sample time, five fish were measured for fork length (where necessary), then sacrificed for pineal dissection. Fish were killed by rapid decapitation and the heads were collected in a foil-covered, ice-filled beaker. All sampling was carried out using only the prevailing lighting condition in the experimental tank at the sampling time (ie. fish sampled during the dark phase were decapitated in the dark). Pineal removal was accomplished as described in Section II.

Heads which were collected in the dark were exposed to white light during pineal removal. To test whether this procedure resulted in changes in HIOMT activity, heads collected during the dark phase were subjected to several treatments. Activities of heads exposed to white light for 30 minutes with pineals removed in white light were compared to activities of heads kept in darkness for 30 minutes with pineals removed in white light and

to activities of heads kept in darkness for 30 minutes with pineals removed in red light (red light was found to have the least effect on HIOMT activity when living rats were subjected to varied wavelengths of light (Cardinali et al., 1972)). No differences were observed among the groups.

Due to instability of the enzyme activity during storage (Table 1), all samples were analyzed immediately following collection.

HIOMT Assay

Since fish availability necessitated the use of a different stock of chinook for these experiments, a check was made of assorted reaction parameters to determine if optimum reaction conditions differed. No difference was found between the Columbia (Tule stock) and Rogue River stocks in any of the parameters tested (pH, temperature, and the effects of NAS and SAM concentration). Therefore, the general assay employed here was the same as described in Section II, with the following exceptions: 1) individual pineals were homogenized in 0.8 ml of a 0.2 M sodium phosphate buffer, pH 7.6; 2) the 12,000 x g centrifugation was eliminated (as previously explained); 3) two assay tubes and a single control tube were run from each homogenate; 4) duplicate aliquots from each homogenate were assayed for total protein (Peterson, 1977); 5) incubation

of samples was for 30 minutes at 30° C. This change was made because HIOMT activities were lower than before (due either to seasonal or size effects on activity) which necessitated the longer incubation time at a higher temperature in order to detect activity.

Lighting Regimes

Experimental tanks were provided with hoods containing two 24" 20-watt Vita-Lite[®] fluorescent bulbs (G.E.) and were made "light-proof" using black 4 ml polyvinyl sheeting. The spectral quality of the lights was tested using a spectroradiometer (ISCO, model SR). No significant difference in spectral quality was detected between the experimental hoods. Diagram 2 shows the arrangement of the experimental room.

Diurnal Cycle (12L:12D): Fish were placed into experimental tanks during normal daylight. Timers were adjusted so tanks were lighted between 8:00 am and 8:00 pm (PDT). Fish were allowed to acclimate to the lighting regime for six days. During the experiment, five fish were sampled every three hours, and pineals were immediately analyzed for total protein and HIOMT activity as previously outlined.

Continuous Light--Continuous Darkness: One tank of fish was set up with lights continuously on, while a

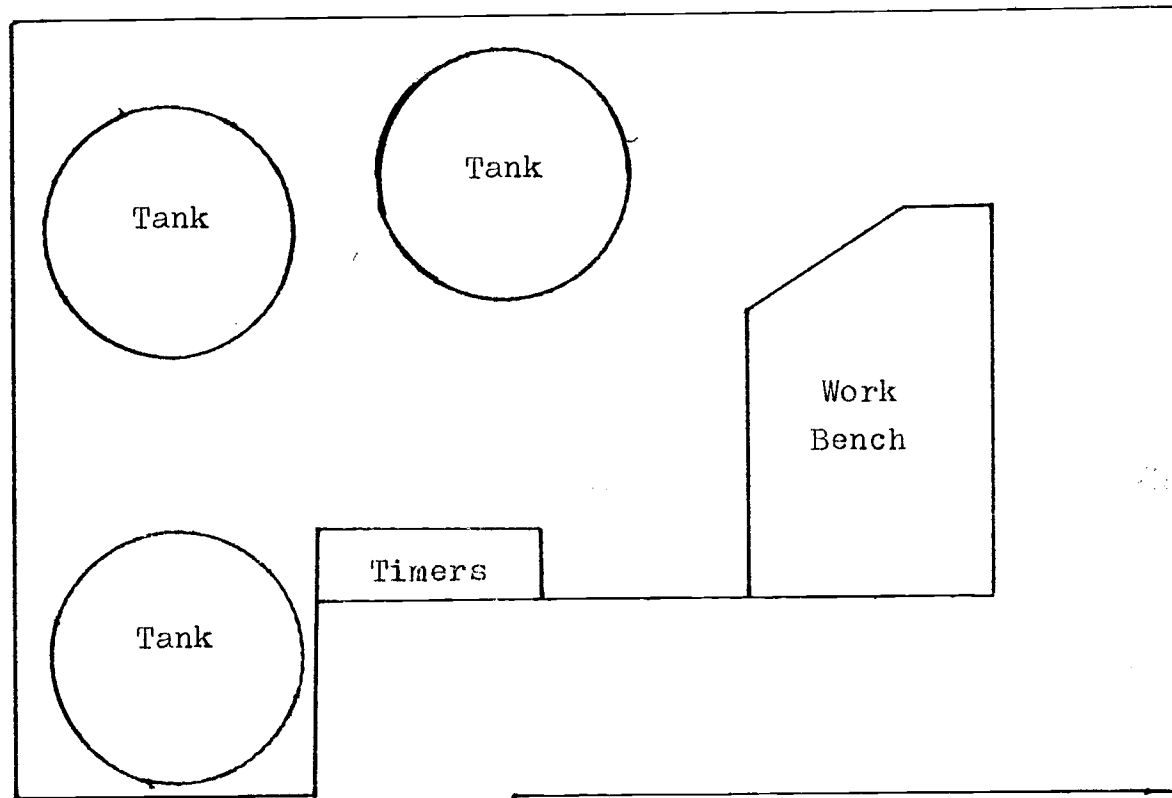


Diagram 2. Diagrammatic representation of room for photoperiod experiments.

second tank received no light. A "control tank" was run simultaneously which had a 12L:12D regime (see above).

"Darkness" within the tanks as well as within the room was checked using 400 ASA photographic film (Kodak Tri-X) and X-ray film (Kodak). Pairs of film strips (one covered by foil the other uncovered) were placed inside the tanks while the room lights were on, to test the "darkness" within the tanks. Film strips were also placed on the work bench of the room while the tank lights were on, to test the "darkness" within the room. The films remained in place for a period of 6 hours. Densities of all uncovered film was the same as that which was covered by foil in all cases. Fish were, again, acclimated to the various lighting regimes for six days prior to sampling. Pineal dissection and HICMT analysis were carried out as described before.

Light Interference: Two identical tanks were established with a 12L:12D photoperiod. Fish in both tanks were acclimated to this photoperiod for six days. On the day of the experiment, the timer for one tank (experimental) was adjusted to give an additional six hours of light at the end of the day, thus effectively providing these fish with eighteen hours of light and six hours of darkness. The timer of the other tank (control) remained set for twelve hours of light and twelve hours of darkness. Sampling and assays were performed as described.

Results

Preliminary experiments indicated that no significant differences in HIOMT specific activity (pmoles melatonin produced/hour/ug protein) could be detected between a peak in activity seen at 11:00 pm and a nadir seen at 11:00 am (Figure 10). In order to minimize variability and thus increase the chances of detecting significant changes in HIOMT specific activity, a series of experiments were performed to examine an apparent relationship between fork length and HIOMT activity (pmoles melatonin produced/hour/pineal). An excellent relationship between fork length and HIOMT activity was found (Figure 11) which fits the equation: $\text{HIOMT activity} = 186 \text{ pmoles/hr/pineal/cm} \times \text{Fork Length} - 1164 \text{ pmoles/hr/pineal}$ ($r^2=0.7157$, $N=50$). This relationship was not evident between HIOMT activity and pineal protein ($r^2=0.3532$, $N=50$). The length vs HIOMT activity relationship prompted the sorting of fish into narrow size classes prior to further experimentation to minimize variability in activity due to differences in fish length.

A diurnal cycle in HIOMT specific activity was seen in multiple repeats of the Diurnal Cycle experiment (see above) using fish that had been size sorted prior to sampling (Figure 12). The peak in specific activity occurred at 11:00 pm and a nadir at 5:00 pm (significantly

Figure 10. Diurnal cycle (12L:12D)--H10MT specific activity. ($\bar{x} \pm$ S.D. N=5) Fish were not size sorted for this experiment. H10MT assay was performed as described in Materials and Methods. Solid bar indicates dark portion of photoperiod.

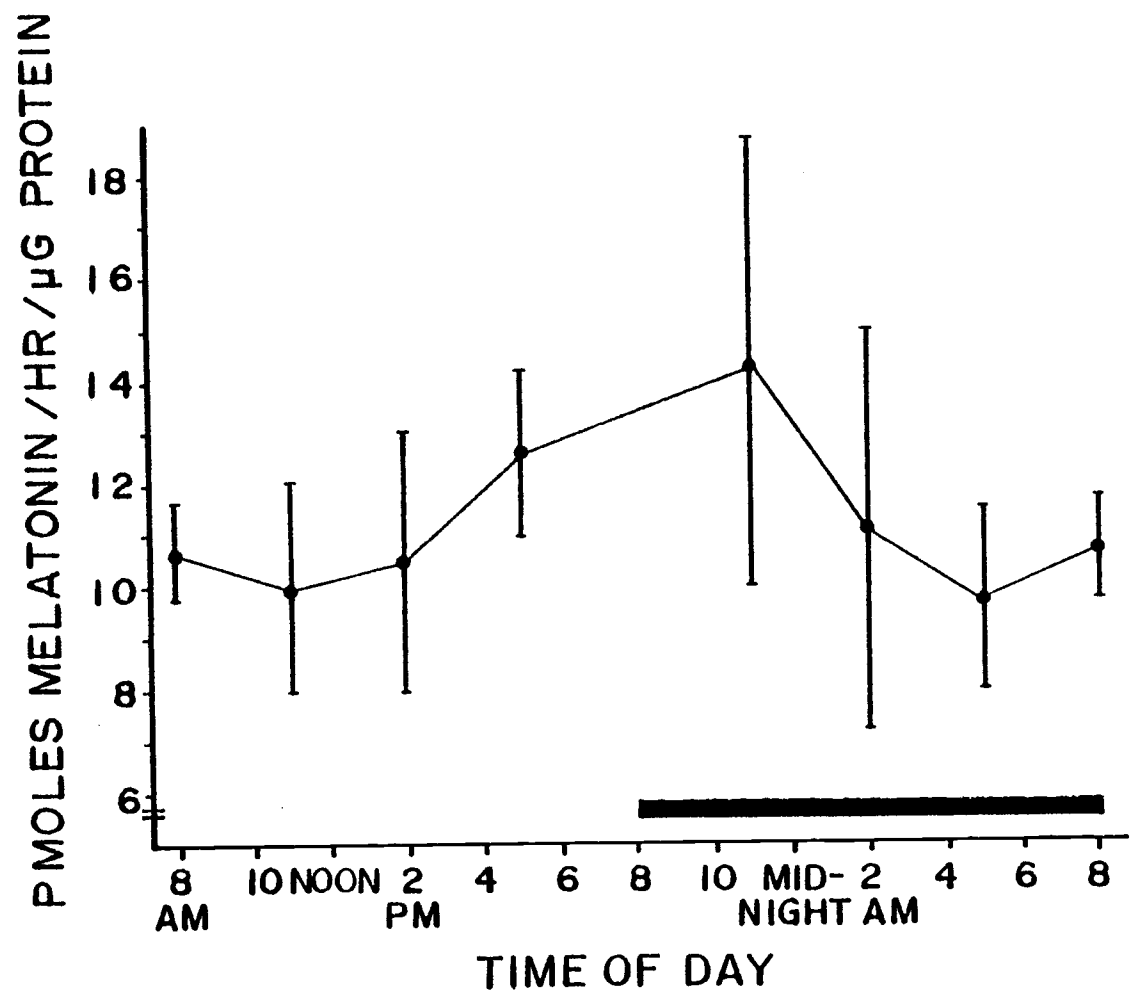


Figure 11. Effect of fish length on melatonin production
by HIOMT. Each point represents a single
pineal.

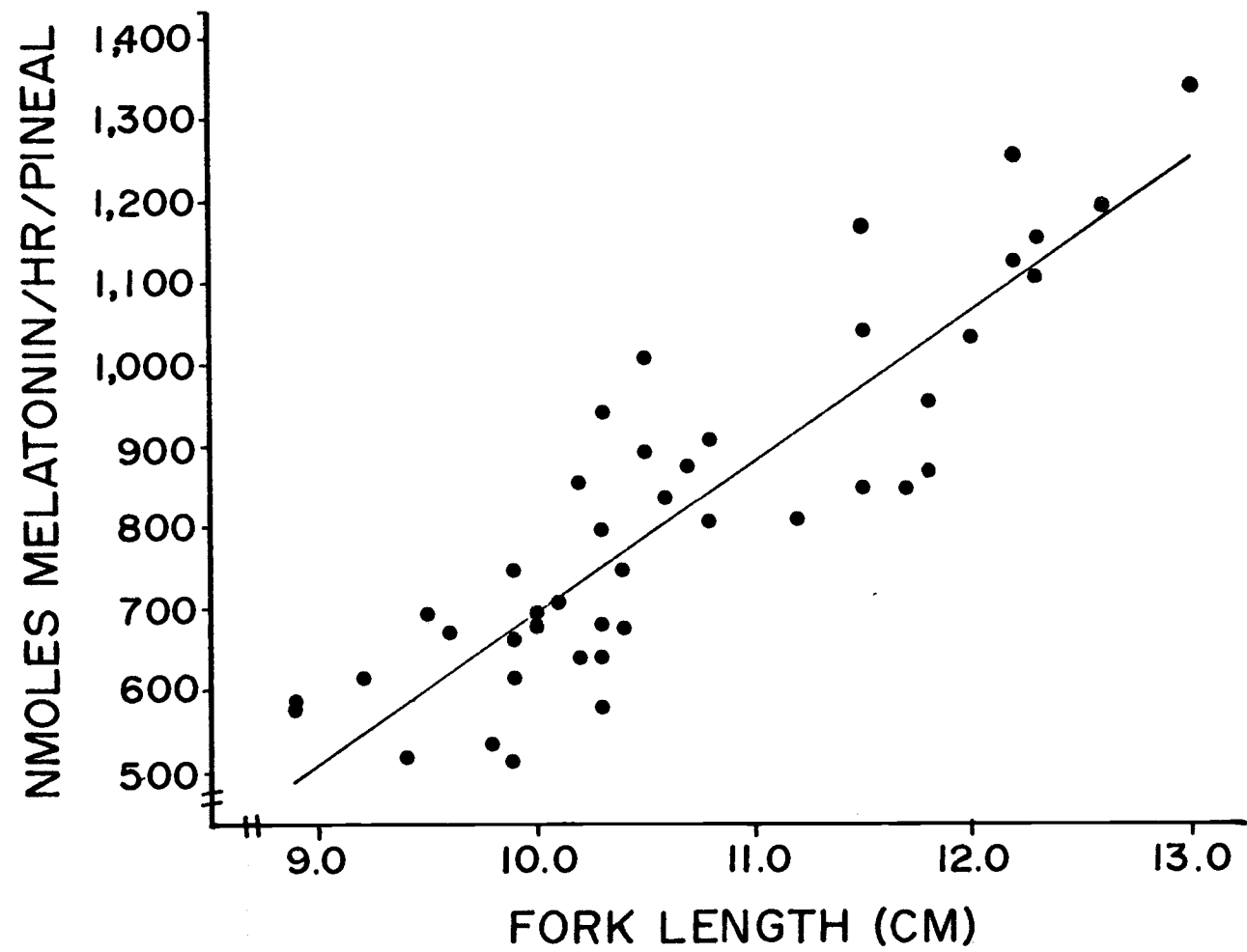
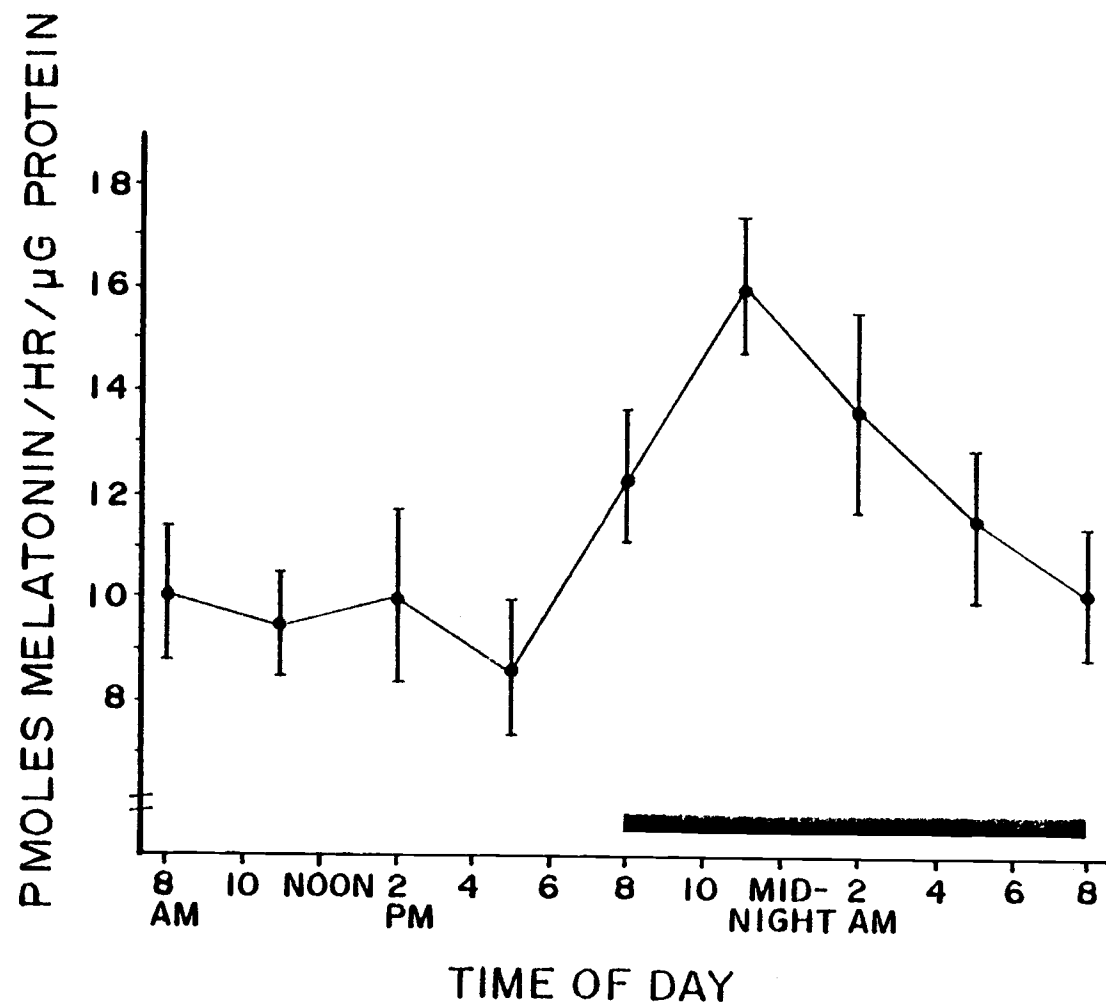


Figure 12. Diurnal cycle (12L:12D)--HIOMT specific activity.
($\bar{x} \pm$ S.D. N=5) Fish were size sorted prior
to the experiment. (11.0-11.5 cm). Solid
bar indicates dark portion of photoperiod.



different, $P < 0.005$), with the increase in activity beginning before the lights went off. Diurnal cycling in specific activity was also seen when fish were subjected to continuous darkness, while no diurnal change was seen with continuous light (Figure 13). When fish acclimated to a 12L:12D photoregime, but sampled with eighteen hours of light and six hours of darkness, were examined, the cycle in specific activity apparently was suppressed (Figure 14). Activity had begun to increase prior to the "expected" dark-time (8:00 pm), however, continued exposure to light prevented the increased specific activity noted during the dark-time in the control group.

The evidence presented for diurnal cycling in HIOMT specific activity in chinook pineals appears to be following the pattern seen in the mammalian system for the biosynthesis of melatonin (Klein, 1978). Although the model proposed by Klein (1978) has best been studied as it pertains to serotonin N-acetyltransferase (see Figure 1) and not HIOMT, the implications are that HIOMT is controlled in a manner similar to serotonin N-acetyltransferase. These control mechanisms involve a proposed endogenous oscillator which increases pineal enzymatic activity during the dark phase of the photoperiod. This dark phase increase in enzyme activity can be blocked by light, however, darkness during the light phase does not increase enzyme activity. The presence of a diurnal cycle in specific

Figure 13. Continuous light-Continuous darkness--
HIOMT specific activity. ($\bar{x} \pm$ S.D. N=5)
 Fish were size sorted prior to the experiment
 with fish sizes ranging from 11.0 to 11.5 cm.
 Solid bar indicates dark portion of photoperiod
 in the control tank (12L:12D). ●—● 12L:12D,
 O----O continuous light, ■---■ continuous
 darkness.

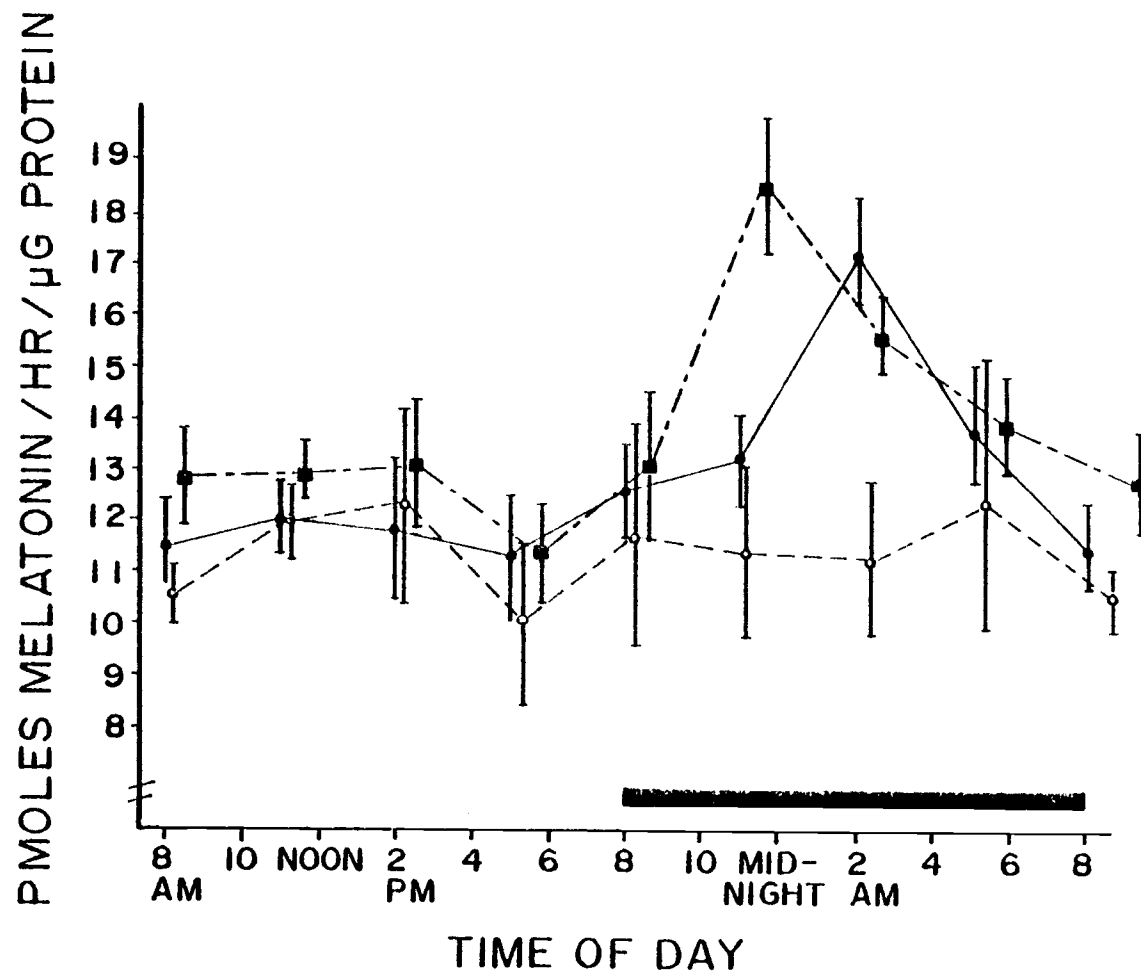
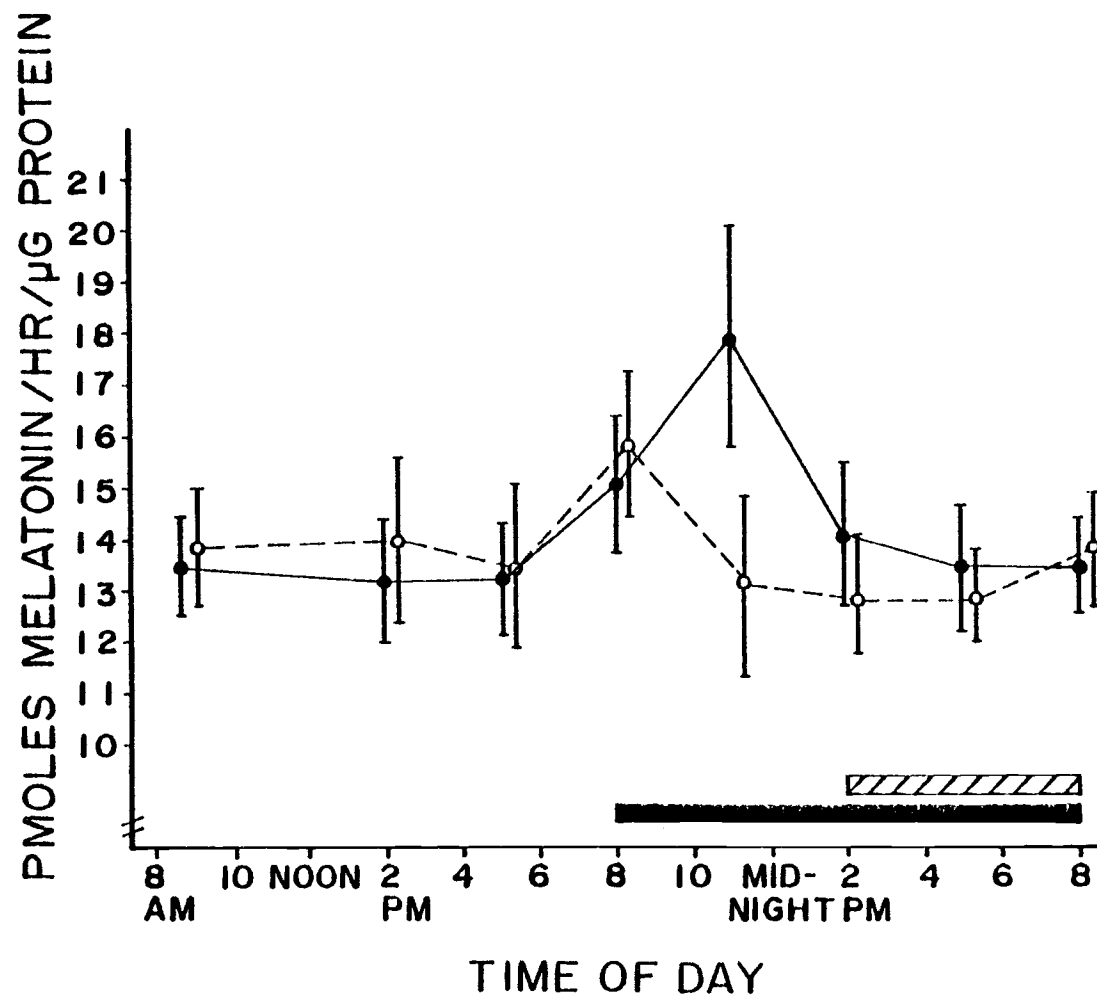


Figure 14. Light interference--HIOMT specific activity.
 Fish size sorted to 11.0-11.5 cm prior to
 the experiment ($\bar{x} \pm S.D.$ N=5). Solid bar
 indicates the dark portion of the photoperiod
 in the control tank (12L:12D), hatched bar
 is the dark portion in the experimental tank
 (18L:6D). ●—● 12L:12D (control),
 O----O 18L:6D (experimental).



activity which persists in continuous darkness, is abolished in continuous light, and is suppressed by extended day-length as found in the present study seems to indicate a similar endogenous oscillator is functioning in the chinook salmon.

Further examination of data from these studies revealed the existence of another diurnal cycle which may be affecting the cycle that was found in specific activity. Figure 15 shows that pineal protein undergoes a diurnal cycle opposite in phase to that of specific activity. Cycling in pineal protein content was seen in all repeats of the Diurnal Cycle experiment, as well as in the control groups (12L:12D) of the other experiments (Figures 16 and 17). Examination of HIOMT activity (pmoles melatonin/hour) (Figures 18, 19, and 20) shows no apparent change regardless of experimental treatment. Thus, it appears quite likely that the diurnal cycling of HIOMT specific activity is merely an artifact of the cycles in pineal protein.

Discussion

Considerable variability in the results of the effects of light on HIOMT activity has been observed in previous studies (Smith and Weber, 1974; Hafeez and Quay, 1970b; Quay, 1967; Moore et al., 1967; Moore, 1977; Wurtman and Mokowitz, 1977). This has led to a controversy as to the role of

Figure 15. Diurnal cycle (12L:12D)--Pineal protein.
Fish size sorted prior to the experiment
(11.0-11.5 cm). Values represent the amount
of protein in a 0.1 ml aliquot from individual
pineal homogenates ($\bar{x} \pm$ S.D. N=5). Solid bar
indicates the dark portion of the photoperiod.

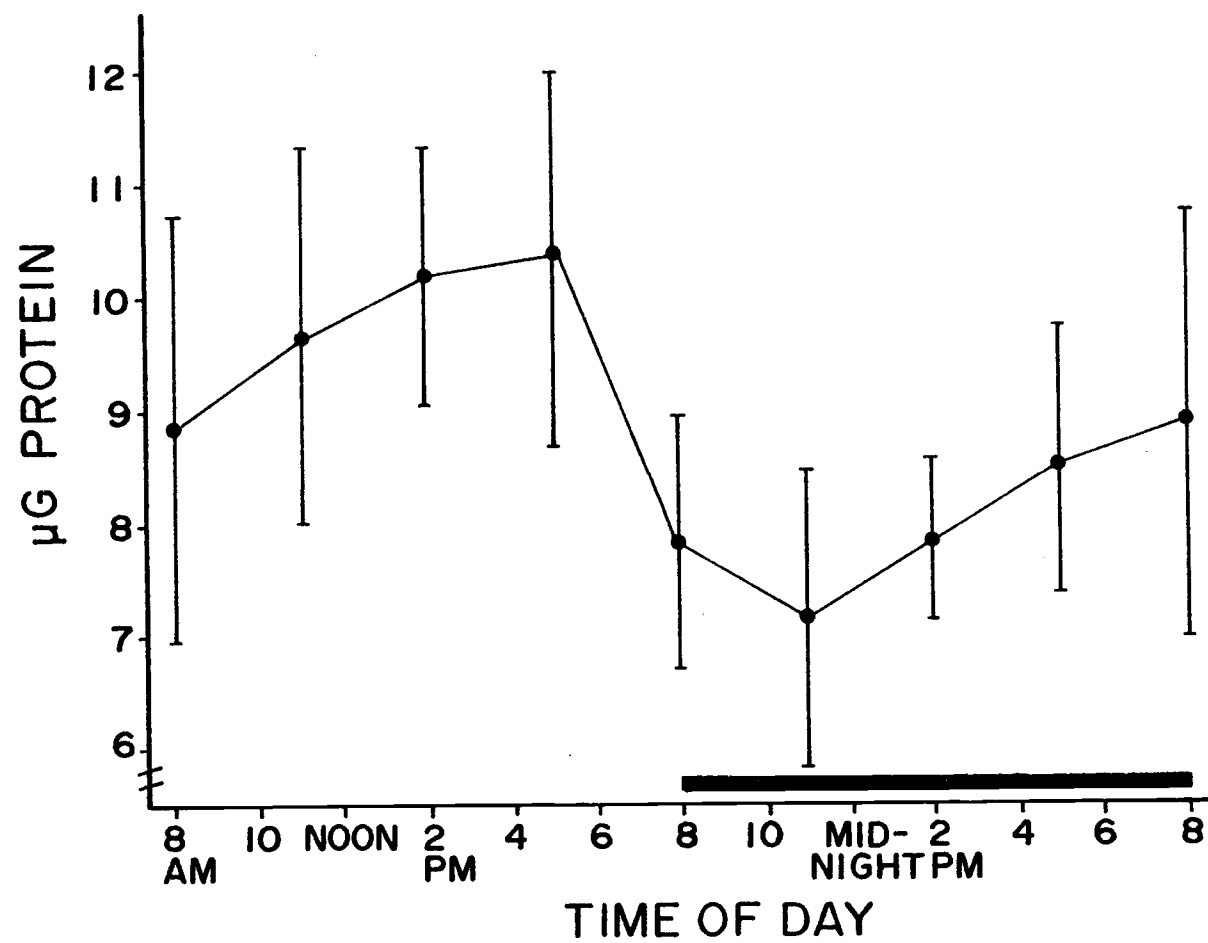


Figure 16. Continuous light-Continuous darkness--
Pineal protein. Fish size sorted prior
to the experiment (11.0-11.5 cm).
Values represent the amount of protein in
a 0.1 ml aliquot from individual pineal
homogenates ($\bar{x} \pm$ S.D. N=5). Solid bar
indicates dark portion of photoperiod in
the control tank (12L:12D). ●—● 12L:12D,
O-----O continuous light, ■---■ continuous
darkness.

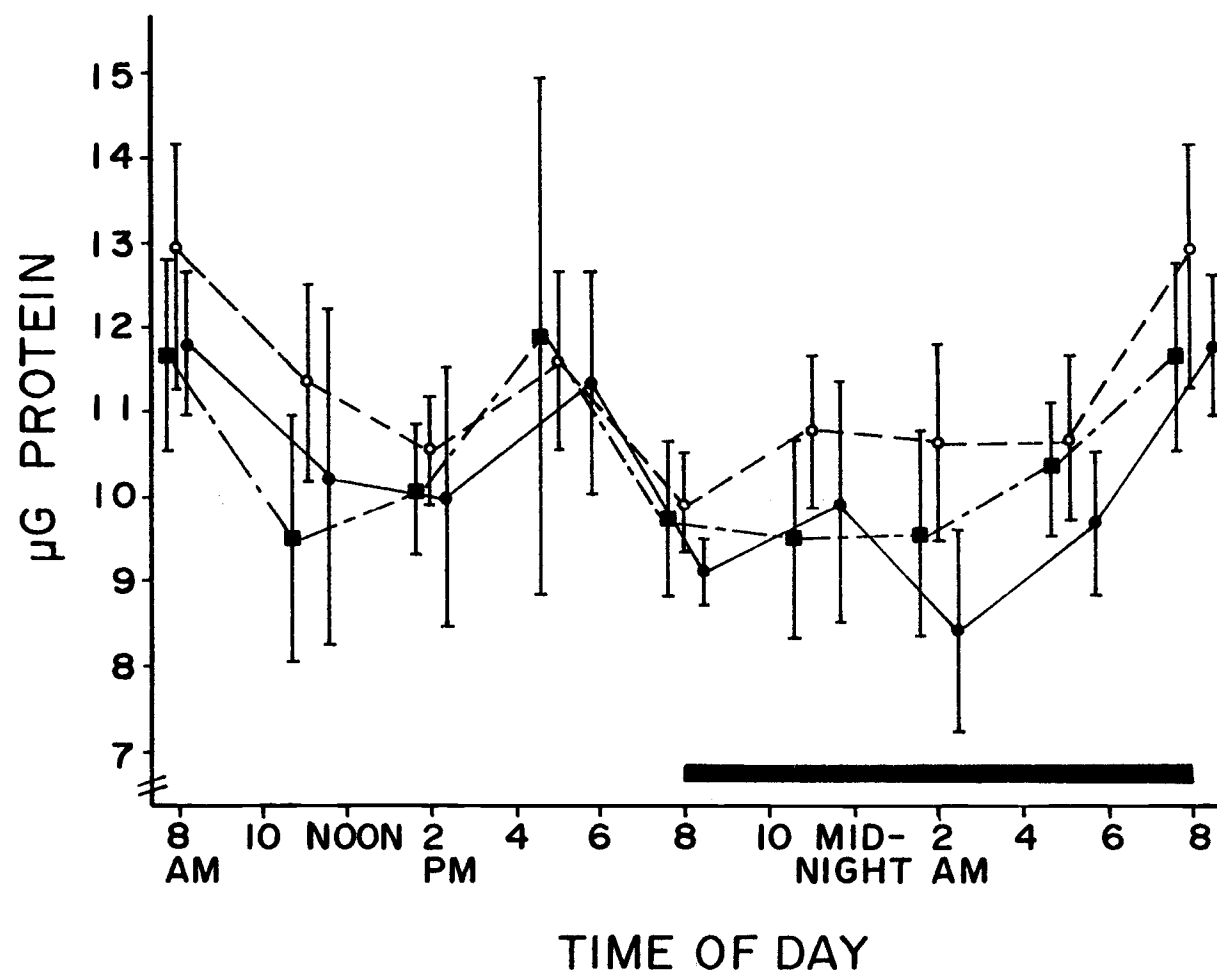


Figure 17. Light interference--Pineal protein. Fish were size sorted prior to the experiment (11.0-11.5 cm). Values represent the amount of protein in a 0.1 ml aliquot from individual pineal homogenates ($\bar{x} \pm S.D.$ N=5). Solid bar indicates dark portion of photoperiod in the control tank (12L:12D), hatched bar is the dark portion in the experimental tank (18L:6D). ●-----● 12L:12D, 0-----0 18L:6D.

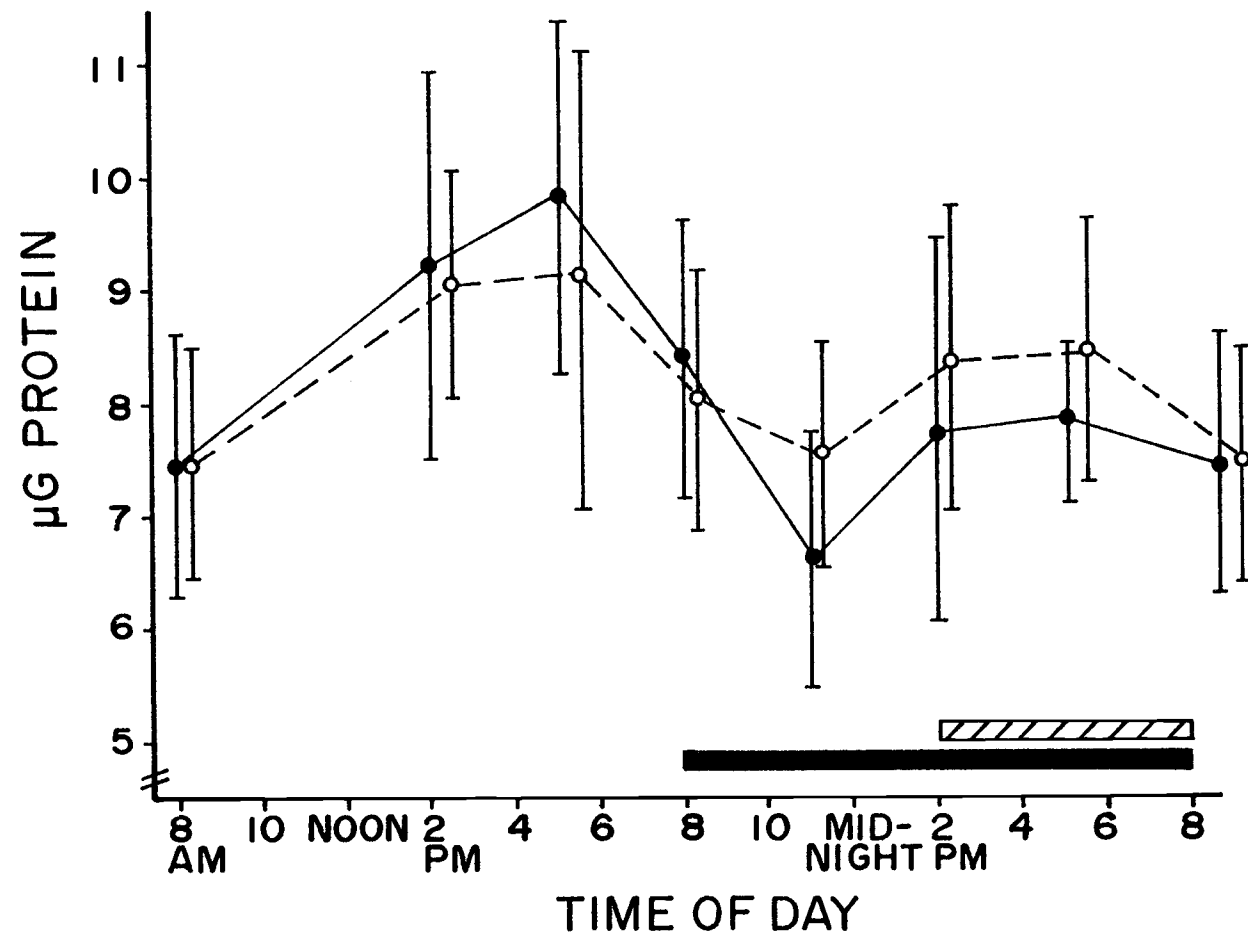


Figure 18. Diurnal cycle (12L:12D)--HIOMT activity.
Fish were size sorted (11.0-11.5 cm) prior to the experiment. Values represent HIOMT activity in a 0.1 ml aliquot from individual pineal homogenates ($\bar{x} \pm$ S.D. N=5). Solid bar indicates dark portion of the photoperiod.

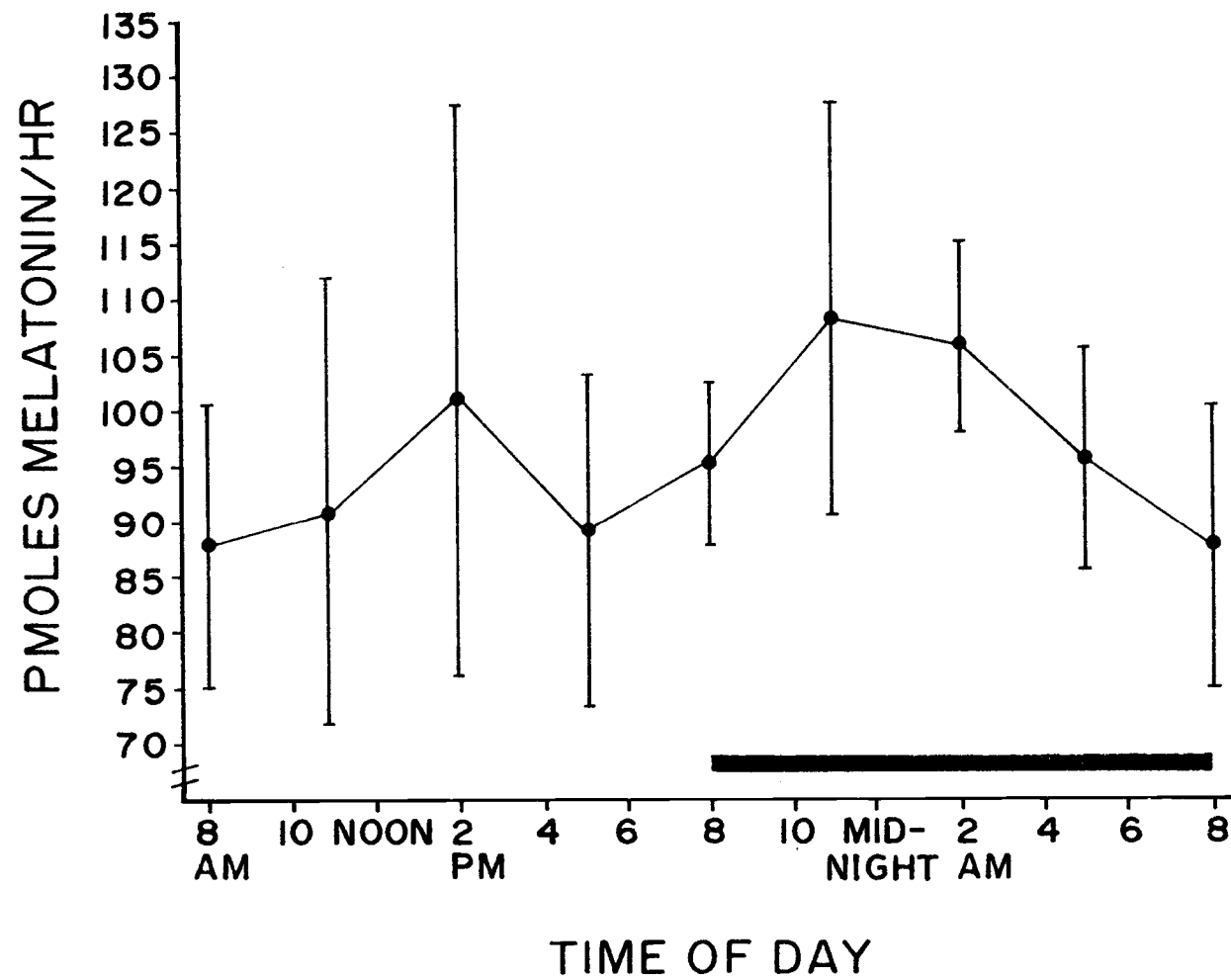


Figure 19. Continuous light-Continuous darkness--HIOMT activity. Fish size 11.0-11.5 cm. Values represent HIOMT activity in a 0.1 ml aliquot from individual pineal homogenates ($\bar{x} \pm S.D.$ N=5). Solid bar indicates dark portion of the photoperiod in the control tank (12L:12D).
●——● 12L:12D, O-----O continuous light,
■---■ continuous darkness.

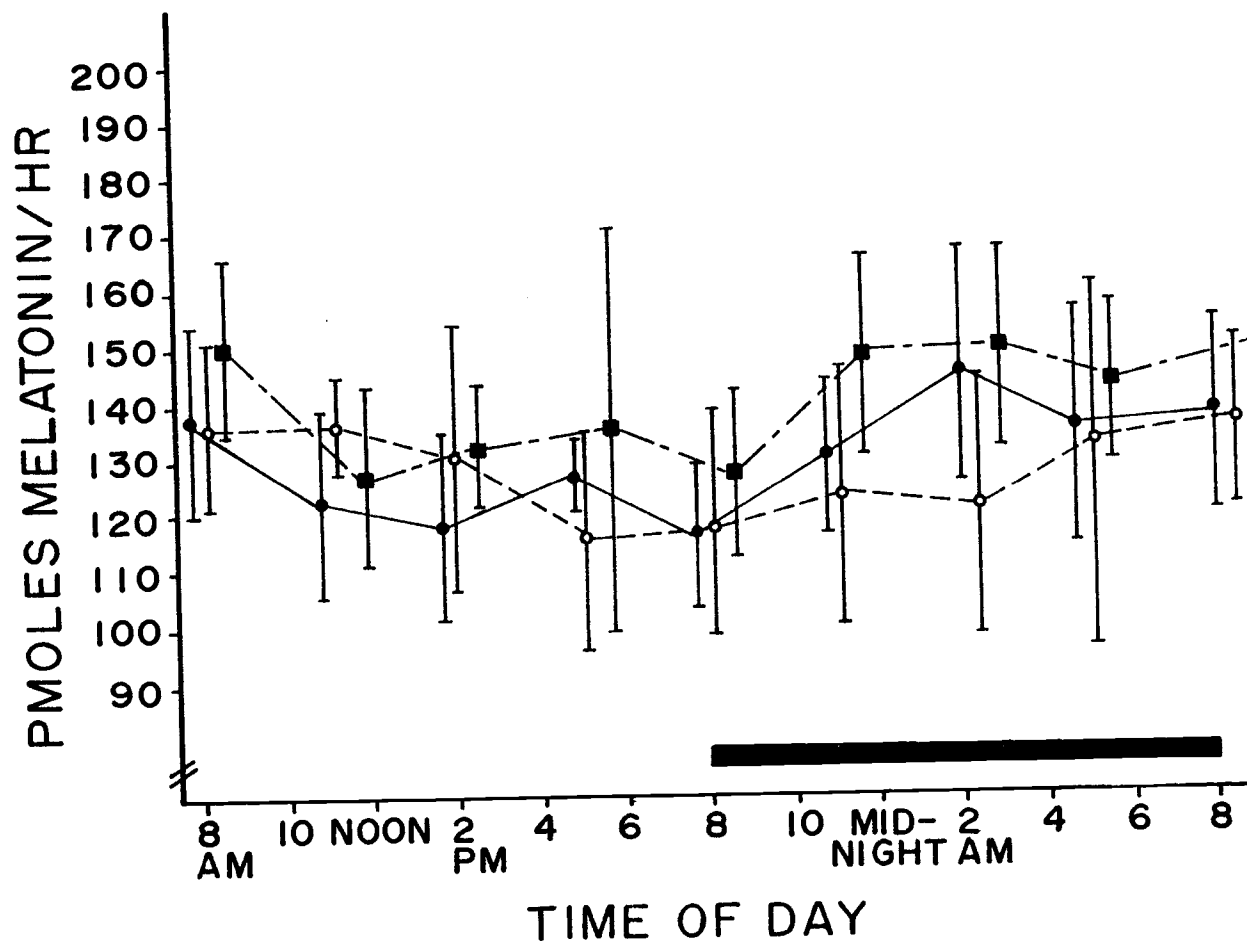
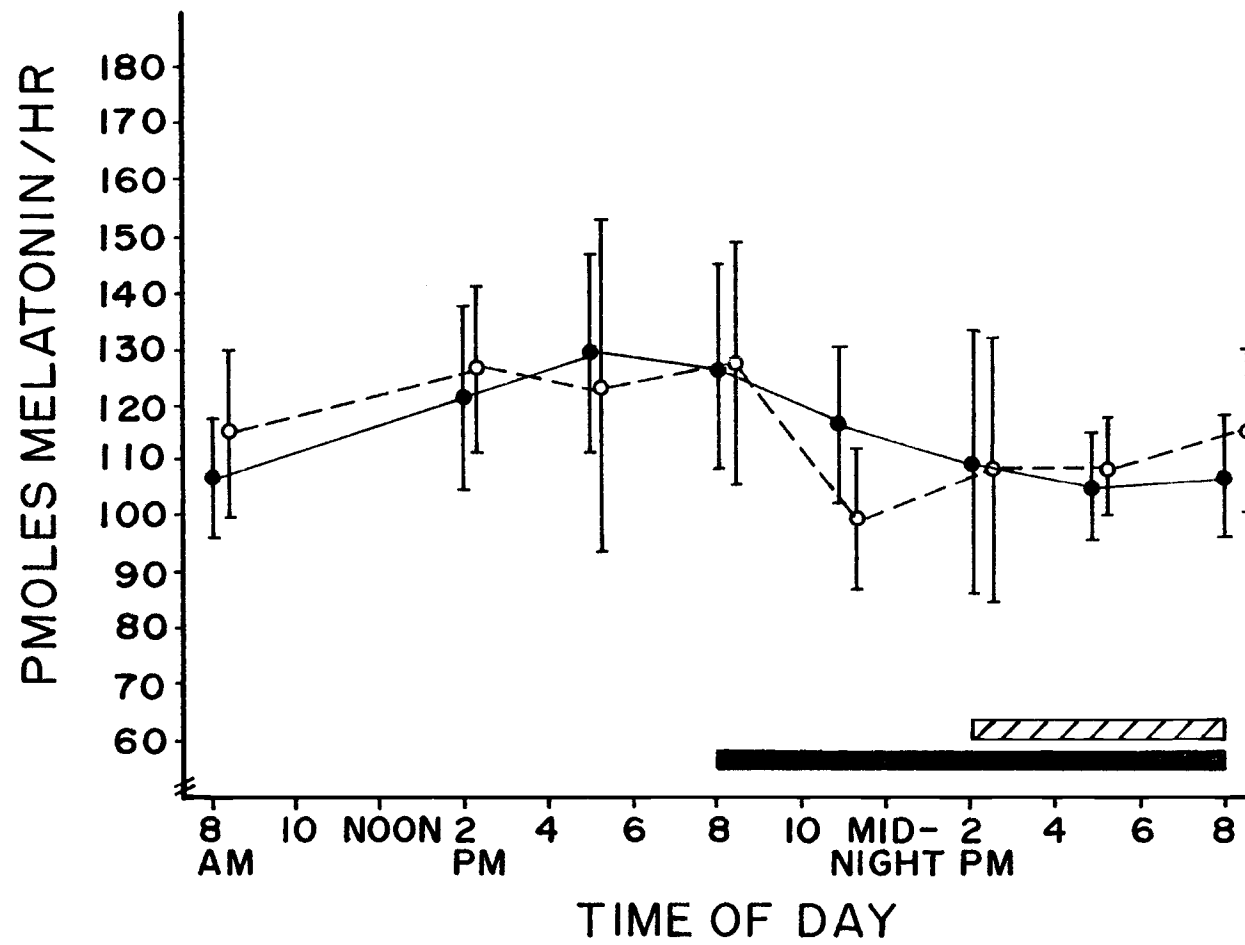


Figure 20. Light interference--HIOMT activity. Fish were sorted to a size range of 11.0 to 11.5 cm prior to the experiment. Values represent HIOMT activity in a 0.1 ml aliquot from individual pineal homogenates ($\bar{x} \pm S.D.$ N=5). Solid bar indicates dark portion of the photoperiod in the control tank (12L:12D), hatched bar is the dark portion of the photoperiod in the experimental tank (18L:6D). ●————● 12L:12D, O-----O 18L:6D.



HIOMT in melatonin production. It was initially postulated that cyclic activity in HIOMT was responsible for diurnal cycles seen in melatonin production (Axelrod et al., 1965b). However, further work has suggested that some factor other than HIOMT may be responsible for cycles seen in melatonin concentration. Pineal HIOMT activity can not consistently be shown to cycle even though investigators have found that plasma and pineal melatonin concentrations do cycle.

Gern et al. (1978), using rainbow trout, have shown that the route by which light affects plasma melatonin levels does not correlate with the route described by Smith (1976) for trout pineal HIOMT activity. Klein (1978) showed a lack of correlation between HIOMT activity and melatonin concentration in rat pineals. These findings suggest the possibility that HIOMT activity is not an accurate reflection of circulating melatonin levels, as previously was felt to be the case.

It is possible that a correlation between HIOMT activity and melatonin levels does exist but the activity of HIOMT measured by the method used does not reflect the cellular activity. The findings of studies performed by Axelrod et al. (1965b) show that HIOMT activity is controlled by changes in protein synthesis. Studies by Jackson and Lovenberg (1971) have shown that in rats, HIOMT comprises almost 4% of the total pineal proteins. As such, they conclude that it would take appreciable changes in protein

synthesis to produce even small changes in HIOMT activity if changes in enzyme quantity were needed to cause the activity changes. Instead, they suggest that aggregation of existing enzyme molecules, predicted from their studies, may account for diurnal fluctuations in HIOMT activity, since the aggregates exhibit differing activities. It may be possible that the effects of changes in protein synthesis seen by Axelrod et al. (1965b) were acting on this association-dissociation of HIOMT aggregates. Further investigation, using techniques which allow accurate determination of enzyme quantities and possible molecular interactions, will be necessary to answer this question.

An alternate explanation for the apparent lack of correlation between melatonin concentration and HIOMT activity can be found in the behavior of the enzyme with differing N-acetylserotonin (NAS) concentrations. Figure 7 shows the effects increasing NAS concentration had on HIOMT activity in this study. Similar results have been found in other studies that have examined this relationship (Quay, 1971; Pelham and Ralph, 1972). The possibility exists that melatonin concentrations can fluctuate as a result of changes in cellular NAS concentration. That is, HIOMT may not be the factor responsible for melatonin fluctuations, but rather changes in NAS concentration as a rate-limiting or inhibiting substrate of HIOMT may cause these fluctuations. Evidence does exist showing

diurnal fluctuations in pineal NAS which supports this contention (Brownstein et al., 1973; Klein and Weller, 1972; Klein, 1978). A review by Klein (1978) examines the possibility that diurnal fluctuations in another enzyme, serotonin N-acetyltransferase (responsible for the formation of N-acetylserotonin (see Figure 1)), leads to the changes seen in NAS and ultimately melatonin. Changes in protein synthesis are implicated here also, but in this case the changes involve control of the synthesis and/or degradation of serotonin N-acetyltransferase.

In the present study, diurnal fluctuations in total pineal protein concentration found in chinook salmon could fit into either of the above schemes. However, the protein changes seen may reflect changes in active pineal factors which are not related to melatonin. Peptide factors from the pineal have been implicated as influencing several physiological processes similar to melatonin (Cheesman, 1970; Pavel and Petrescu, 1966; Benson et al., 1971, 1972; Blask et al., 1976). Thus, it appears possible that some of the diurnal or seasonal cycles previously felt to be modulated by melatonin may in fact be controlled by pineal protein factors.

Diurnal fluctuations in rat pineal proteins, similar to those found in chinook salmon in the present study, have been shown (Nir et al., 1973). Peak protein concentrations occurred during daylight and low values occurred at

night. Nir et al. (1973) were also able to show that two major protein components exhibited diurnal fluctuations 180° out of phase with each other. Further research will be necessary to determine the significance of these findings.

The data obtained in the present study on the effects of light on HIOMT activity indicate that the activity in chinook salmon does not exhibit true diurnal fluctuation. The apparent diurnal cycling seen in HIOMT specific activity merely reflects changes in pineal protein concentration and not changes in HIOMT activity as measured in this system.

IV. SUMMARY

It was the intent of this thesis to study the effects of light on pineal HIOMT activity in chinook salmon (Oncorhynchus tshawytscha). Since previous investigators had shown that species differences exist in the reaction characteristics of HIOMT, the kinetics of the enzyme from chinook were examined to optimize reaction parameters before utilizing the assay to study light effects on the enzyme.

The most applicable conditions for routine analysis of HIOMT from chinook pineals were found to be as follows: Assay immediately following sacrifice of fish, homogenize pineals in 0.2 M sodium phosphate buffer, pH 7.6, NAS concentration of 5.0×10^{-6} M, SAM concentration of 1.1×10^{-5} M (concentration are those in reaction mixture), incubation temperature of between 20° and 30°C, and a reaction time of less than 60 minutes. With the exception of pH and effects of NAS, characteristics of chinook pineal HIOMT are similar to those of most other vertebrates.

Anatomical localization studies indicated that HIOMT activity can be found in the pineal, retina, and dorsal diencephalon. However, the activity in the pineal is significantly higher than that in the other tissues. Pineal HIOMT activity is localized subcellularly in the

post-microsomal supernatant fraction (100,000 x g supernatant). The anatomical and subcellular localization of the enzyme is comparable to findings in other vertebrates.

Studies on the effects of light on HIOMT activity revealed a diurnal cycle in specific activity (pmoles melatonin produced/hour/ug protein). This cycle continued in continuous darkness but was abolished by continuous light or interference by light during the dark-time. No diurnal cycles in HIOMT activity (pmoles melatonin produced/hour) were apparent in any of the lighting regimes. Pineal protein content, though, did show diurnal fluctuations. The evidence presented points to the conclusion that cycles in specific activity simply reflect the protein cycles and as such are artifacts in this system.

While no diurnal cycle in HIOMT activity was found in chinook pineals, preliminary findings indicate that a seasonal cycle may exist. Seasonal cycles in HIOMT were postulated by Klein (1978) and could represent an important regulatory mechanism for controlling seasonal changes in melatonin production, and thus, be a key to the mysteries of the control of many physiological processes which show seasonality.

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