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Title: PINEAL AND RETINAL ACETYLSEROTONIN METHYLTRANSFERASE ACTIVITY IN TROUT

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Pineal acetylserotonin methyltransferase (ASMT) activities in rainbow trout were reported on by Hafeez and Quay (1971). The validity of certain assay parameters used by these researchers were investigated. With the exception of incubation temperature, all parameters studied proved to be quite valid. Temperature studies indicated a low stability for trout ASMT at 40°C. At this temperature enzymatic activity ceased after 20 minutes of incubation.

Using the assay parameters developed from the previous experiments, levels of retinal ASMT were monitored in mature kamloops rainbow trout. Retinal ASMT activities of pinealectomized and sham-pinealectomized fish displayed no differences from the retinal ASMT activity of untreated controls.

The same assay system was again utilized to investigate the possible diurnal ASMT activity changes in the pineal of steelhead

trout smolts. A diurnal fluctuation in ASMT levels was discernible when total pineal protein was used to standardize the data. Although a similar trend could be seen when ASMT activity was expressed as a function of mean body weight, the pattern was not as consistent.

Pineal and Retinal Acetylserotonin Methyltransferase Activity in Trout

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INTRODUCTION

While the pineal gland has recently been the subject of several intensive studies, interest in it has its roots in antiquity. The "little pine cone," as it was known to the Greeks, was believed to control the flow of thoughts in the mind, and Descartes hypothesized that it was the seat of the soul. The pineal remained shrouded in myth until the turn of the century when Heubner (1898), McCord and Allen (1917), and Holmgren (1918), using more modern techniques, began investigating the gland with greater objectivity.

McCord and Allen (1917) demonstrated a dramatic blanching response of frog skin to bovine pineal extract. Other investigators recognized photoreceptor organelles in the pineals of lower vertebrates (Dodt, 1963; Morita, 1966). Lerner (1958) isolated the "blanching principle" from bovine pineal extracts and found it to be 5-methoxy-N-acetyltryptamine, which was named melatonin. Later, neurophysiologists showed that the pineals of lower vertebrates could respond to light of different wavelengths by sending nervous impulses down the pineal stalk to the brain (Dodt, 1963).

The isolation of melatonin created further interest in pineal research, which included teleost research also; however, early results were often confusing due to failure to account for species differences and certain environmental factors. Recognizing that overzealous extrapolation from mammals to fish can be misleading, the main impetus for this study comes from a number of independent studies in the fields of comparative physiology and mammalian pineology.

Researchers have demonstrated in mammals that melatonin can inhibit thyroid function by reducing iodine uptake and lowering plasma levels of thyroid stimulating hormone (Panda and Turner, 1968; Bashieri et al., 1963; De Prospo, De Martino and McGuinness, 1968). Studies involving migratory species of sticklebacks and Pacific salmon show that thyroid activity can be associated with saltwater-freshwater preference, which is implicated in upstream and downstream migration (Baggerman, 1963). Furthermore, thyroid function can also affect locomotor activity, growth and maturation, all of which are in some way linked to the migration of fish (Hoar, 1963; Baggerman, 1957, 1959, 1960, 1962).

Also associated with migration in diadromous fish is the modification of osmoregulatory function. Prolactin plays an important role in osmoregulation in teleosts (Pickford and Phillips, 1959; Ball and Olivereau, 1964), and in mammals plasma levels of this

hormone may be, in some way, controlled by the pineal gland (Kamberi, Mical and Parter, 1971; Relkin, 1972).

It becomes apparent that there are implications that the pineal gland of teleosts may serve an important role in the transformation of information from environmental lighting into migratory and reproductive information via nervous and endocrine systems. Since the teleost pineal contains functional photoreceptive elements and has nerve tracts to the diencephalon, it would seem a prime candidate for such a role. Also, the effects of melatonin upon hormones known to be involved with migratory behavior implicate the pineal in a role of neurochemical transducer. Diurnal and seasonal fluctuations in the enzyme, acetylserotonin methyltransferase (ASMT), have been demonstrated in mammals (Axelrod, 1965; Keyes, 1971). This enzyme is responsible for the final step in the production of melatonin. The diurnal change is sensitive to photoperiod, while, in the case of seasonal flux, sensitivity to lighting is strongly suggested (Axelrod, 1965; Keyes, 1971).

The purpose of this study is threefold; (1) to establish an assay system capable of measuring activity of teleost ASMT; (2) to use this assay to determine if pineal ASMT levels fluctuate on a diurnal basis in winter steelhead trout juveniles (Salmo gairdneri); (3) to use this assay to detect retinal ASMT in trout and establish if pinealectomy causes a compensatory change in retinal ASMT content.

METHODS AND MATERIALS

The procedure employed for the assay of ASMT activity was a modification of a procedure previously described by Hafeez and Quay (1970). It relies upon the formation of ^{14}C-melatonin when pineal homogenate is incubated with N-acetylserotonin and ^{14}C(methyl)-S-adenosylmethionine. The fish were sacrificed by severing the hemal canal, after which the top of the skull was excised with scissors along a longitudinal line in the plane of the middle of the eye. The pineals were removed from the excised skull fragments and homogenized in 0.35 ml of a sodium phosphate buffer, pH 7.9. Samples were kept at 4°C until incubation. A ground-glass, hand-operated tissue grinder was employed for all homogenizations. Aliquants (0.1 ml) of homogenate were transferred to two, 15 ml, glass-stoppered centrifuge tubes, and 50 μg of N-acetylserotonin (Regis Chemical Co., Chicago) in 0.05 ml of solution was added to one of the centrifuge tubes. This was followed by 50 mμCi of ^{14}C(methyl)-S-adenosylmethionine (48 mCi/mmole, Amersham/Searle, Arlington Heights) in 0.05 ml of solution. After incubation for 15 minutes at 25°C, 0.35 ml of a 0.2M borate buffer, pH 10.0, and 2.5 ml of a 80%-20% solution of toluene-isoamyl alcohol were added to the centrifuge tube. The tube was shaken by hand for two minutes and the particulate matter was separated

from the supernatant by centrifugation. Two milliliters of the organic phase were transferred to a scintillation vial and one milliliter of 95% ethanol and 10 ml of liquid scintillation fluid (339.6 g naphthalene, 24.75 g diphenyloxalzole, 0.3081 g dimethyl POPOP, three liters of 1, 4-dioxane) were added. All counting was done on a Packard Tri-Carb liquid scintillation counter (model 3380). The second aliquant received 0.05 ml of doubly-distilled water instead of the N-acetylserotonin, and was run concurrently with the other tube as a control.

Student t-tests were used in all cases where statistical analysis was utilized. Identification of labeled melatonin was achieved by thin layer chromatography as described by Klein and Notides (1959).

Buffer Molarity and Trout ASMT Activity

In this study kamloops rainbow trout were used to test the effects of incubation buffer molarity upon the activity of pineal ASMT.

Glands were removed, weighed, and homogenized in a phosphate buffer of one of the following molarities: 0.01, 0.05, 0.1, 0.15, 0.2. Assay procedures were followed as outlined above with three exceptions: (1) incubation temperature was 20^{o}C, (2) the extraction buffer was a 0.5M phosphate buffer, (3) incubation

lasted one hour.

Buffer pH and Trout ASMT Activity

The great physiological differences between trout and mammals prompted this study, aimed at determining an optimal incubation pH for trout ASMT.

Eight pineals from kamloops rainbow trout were initially pooled and homogenized in doubly-distilled water. Three or four 0.07 ml aliquants were drawn off and each received 0.25 ml of a 0.1M phosphate buffer of a certain pH. The pH range was from 7.56 to 8.27. The assay was performed with the modifications listed in the molarity study.

Freezing and Trout ASMT Activity

Time limitations dictated that the glands be frozen for a period of time, which necessitated this investigation into freezing effects.

Pineals of kamloops rainbow trout were pooled and homogenized in three milliliters of 0.2M phosphate buffer. Five 0.5 ml aliquants were transferred to five separate 15 ml centrifuge tubes and frozen. A sixth aliquant was further divided into 0.1 ml samples and assayed accordingly with the same modifications used in the molarity study. On designated days after freezing, the other five

samples were analyzed similarly. Two homogenates were handled in this manner.

Incubation Temperature and Steelhead ASMT Activity

Natural environmental temperatures encountered by trout are considerably different from those seen by mammals, which suggests that trout ASMT activity might have a different temperature optimum and stability.

Ten to fifteen pineals from steelhead trout juveniles were pooled and homogenized. At each of the six temperatures studied ($15^{o}C$, $20^{o}C$, $25^{o}C$, $30^{o}C$, $35^{o}C$, $40^{o}C$) eight 0.1 ml aliquants were used. Four of these eight (including one control) received all substrates and were immediately incubated for ten minutes to determine initial activity. The remaining four samples were preincubated for one hour. After preincubation, substrates were added and samples were incubated an additional ten minutes at the same temperature by using a stability ratio, i.e., the activity in disintegrations per minute (DPM) of the preincubated samples over the DPM of the non-preincubated samples.

The point at which enzymatic activity began to decline was determined by pooling 10-11 glands and incubating 30 aliquants (0.1 ml) at each of the temperatures listed previously. Every ten minutes over a one-hour incubation period, five samples were

removed from incubation and assayed.

Buffer Type and Trout ASMT Activity

Three different buffers with similar buffering ranges were studied in order to establish the most effective buffer for trout ASMT assays.

Fifteen pineals from kamloops rainbow trout were pooled and homogenized in doubly-distilled water. Aliquants (0.05 ml) were placed in 0.25 ml of either Tris, phosphate, or sodium barbital buffers of pH 7.9. Using the modifications listed in the molarity study, with the exception that the extraction buffer was a 0.1M sodium bicarbonate buffer, assays were carried out and activities recorded for the three different buffers.

Diurnal ASMT Activity in Steelhead Pineals

After assay parameters were established, diurnal fluctuations in steelhead pineal ASMT were investigated.

Steelhead trout smolts were sacrificed, weighed, and skull fragments were excised as described earlier. Fragments from two fish were pooled as a single sample and rapidly frozen. Five pooled samples were taken every four hours over a 24 hour period, and on five separate days over a one month period. The extraction of pineals and actual assays were conducted on the two days

following the collection of the samples. Total protein was determined by the Folin phenol technique of Lowry (1951).

Pinealectomy and Retinal ASMT

The effects of pineal removal upon retinal levels of trout ASMT were tested in this study.

Kamloops rainbow trout were used for this experiment. Three fish were pinealectomized, three were sham-pinealectomized, and two were left untreated. Following surgery, all fish were maintained in a dark holding tank at 10-12^{o}C for about four weeks. After the holding period, the pineal and one retina from each fish were removed and assayed, using the procedure discussed in the molarity study. In the case of the pinealectomized trout, tissue around the pineal region was used for the assay.

RESULTS

Molarity Studies

The function of buffer molarity on the ability of trout ASMT to produce melatonin is illustrated in Figure 1. No significant difference could be found among the different molarities, although the 0.2M buffer showed far less variation.

pH Studies

The optimal pH reported for mammalian ASMT activity *in vitro* is 7.9. This study tested the optimal pH for trout ASMT. As indicated in Figure 2, optimal activity was achieved at pH 7.9, although statistically, at the 95% confidence level, only pH 7.56 could be distinguished from 7.9.

Freezing Studies

The necessity of having to freeze the glands because of time considerations prompted this study of the freezing effects upon trout ASMT activity. Figure 3 shows that very little change in enzymatic activity occurred for the first three or four days, after which the two homogenates deviated considerably.

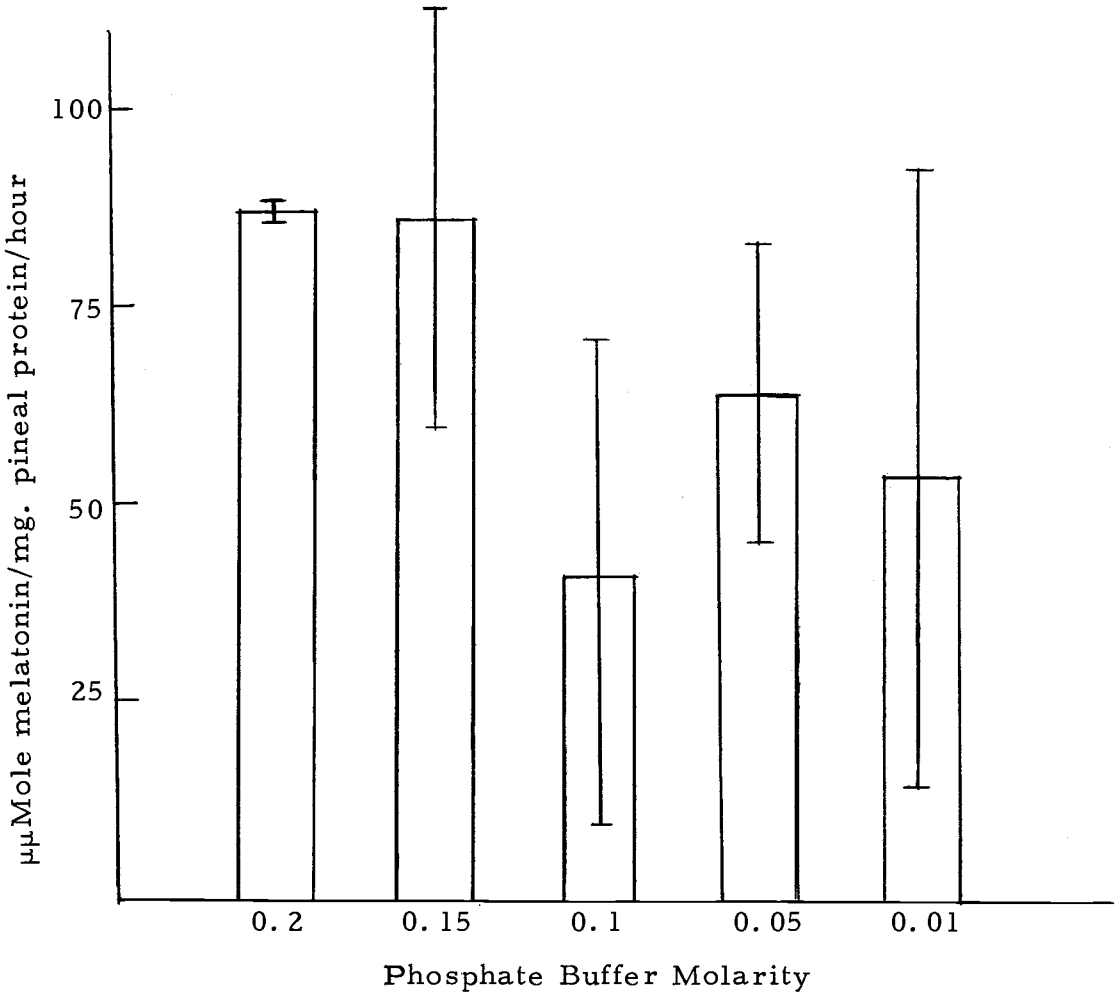

Figure 1. Melatonin production is expressed as a function of phosphate buffer molarity. Mean production ± s.d. is denoted by the columns.

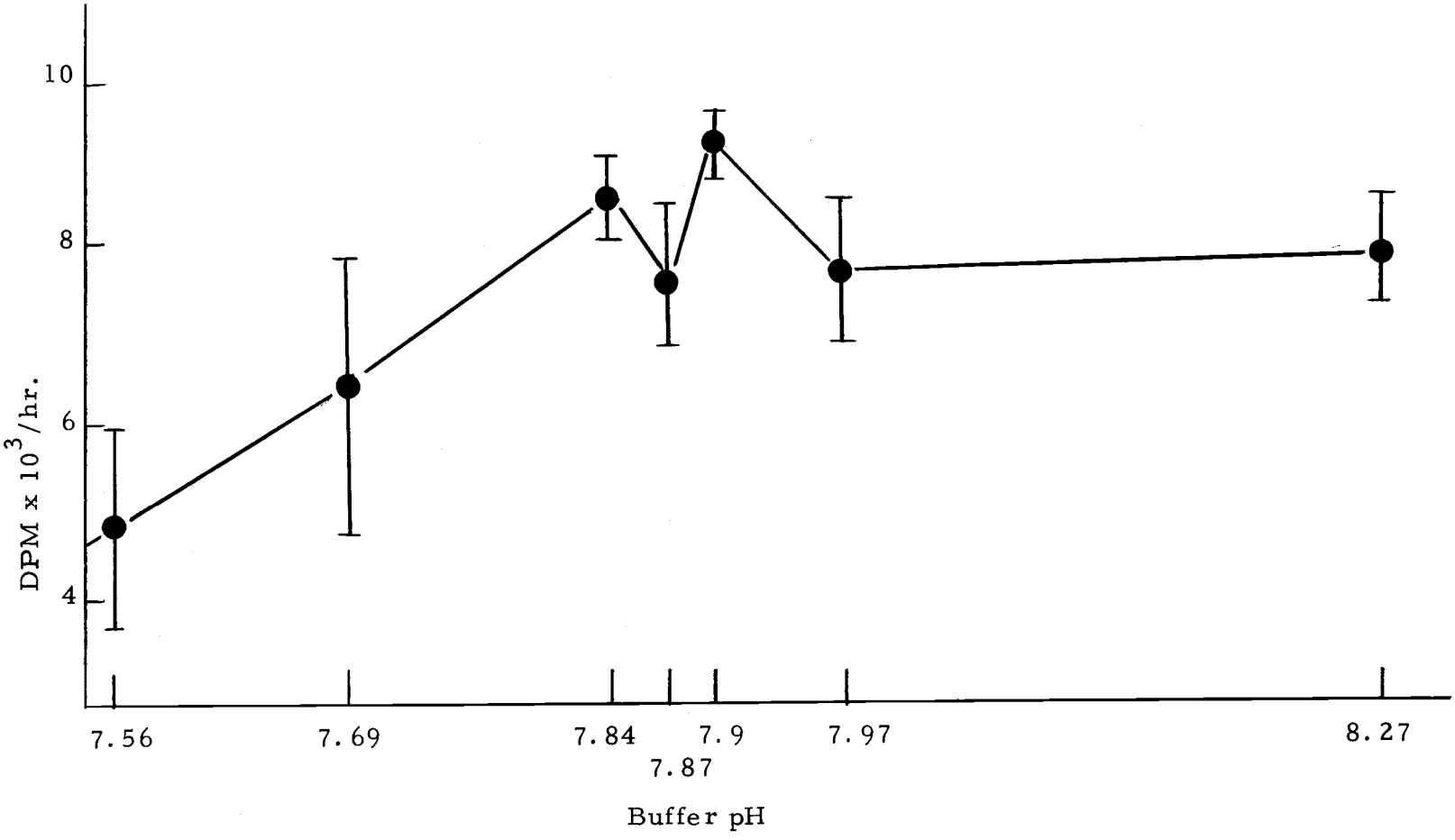

Figure 2. Mean ASMT activity ± s.d. of pineal homogenates in DPM's is expressed as a function of incubation buffer pH

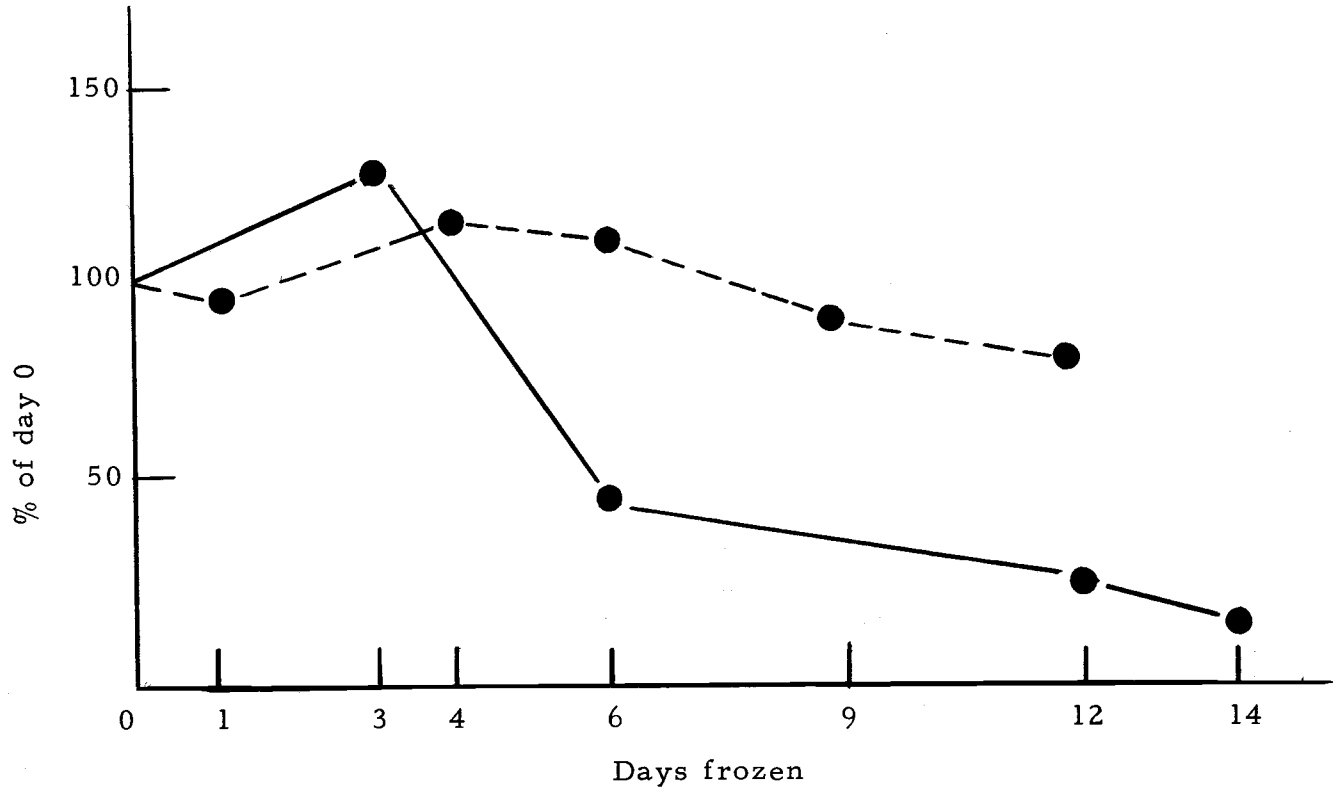

Figure 3. ASMT activity of two different pineal homogenates is expressed as a percentage of initial activity, i.e., activity before freezing. The abscissa represents the number of days samples of homogenates remained frozen before being assayed.

Temperature Studies

Mammalian ASMT requires 37°C for optimal activity in vitro, however, steelhead, in nature, cannot tolerate this temperature. An incubation temperature for optimal stability as well as adequate activity was desired. The results of this study can be seen in Figure 4. The stability at 25°C was significantly ($P < .05$) greater than at 35°C or 40°C, and at the same time, statistically identical to results recorded at 15°C and 20°C. The 30°C temperature was unacceptable due to the tremendous variation associated with the data at this point.

Figure 5 illustrates the point at which steelhead ASMT activity changes over a one hour incubation at different temperatures. The only dramatic depression of enzymatic activity occurred at 40°C and 35°C with all other temperatures showing constant enzymatic activity over the full hour.

Buffer Type Studies

The effect on trout ASMT activity of different buffer moities was evaluated in this study. Figure 6 depicts the results. The enzymatic activity observed using the Tris buffer was significantly less than activities recorded when phosphate or sodium barbital buffers were used. The latter two showed similar results.

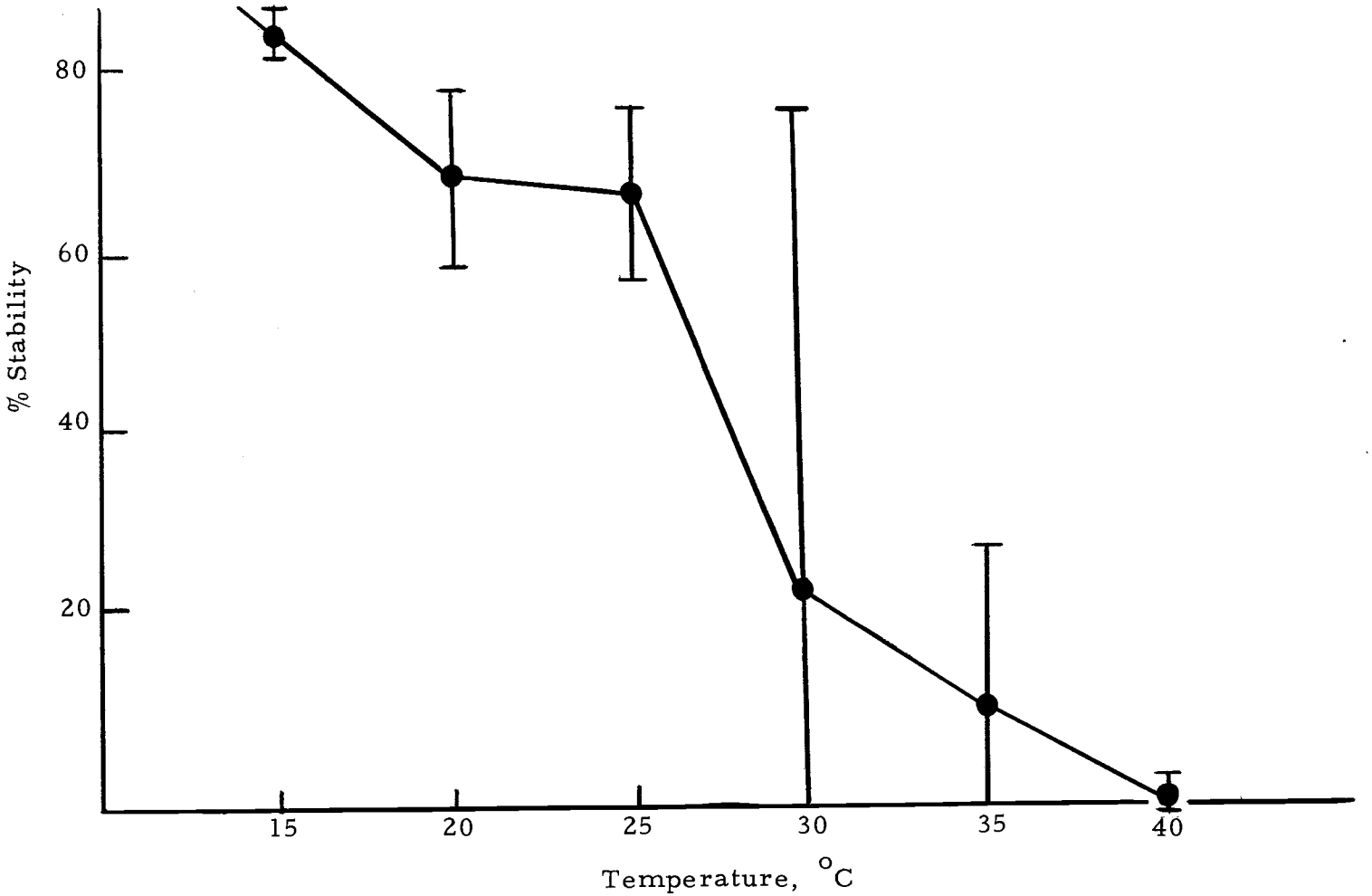

Figure 4. ASMT stability is plotted against temperature. The percentage represents the 10 minute activity following a one hour preincubation divided by the initial 10 minute activity. All points denote mean values ± s. d.

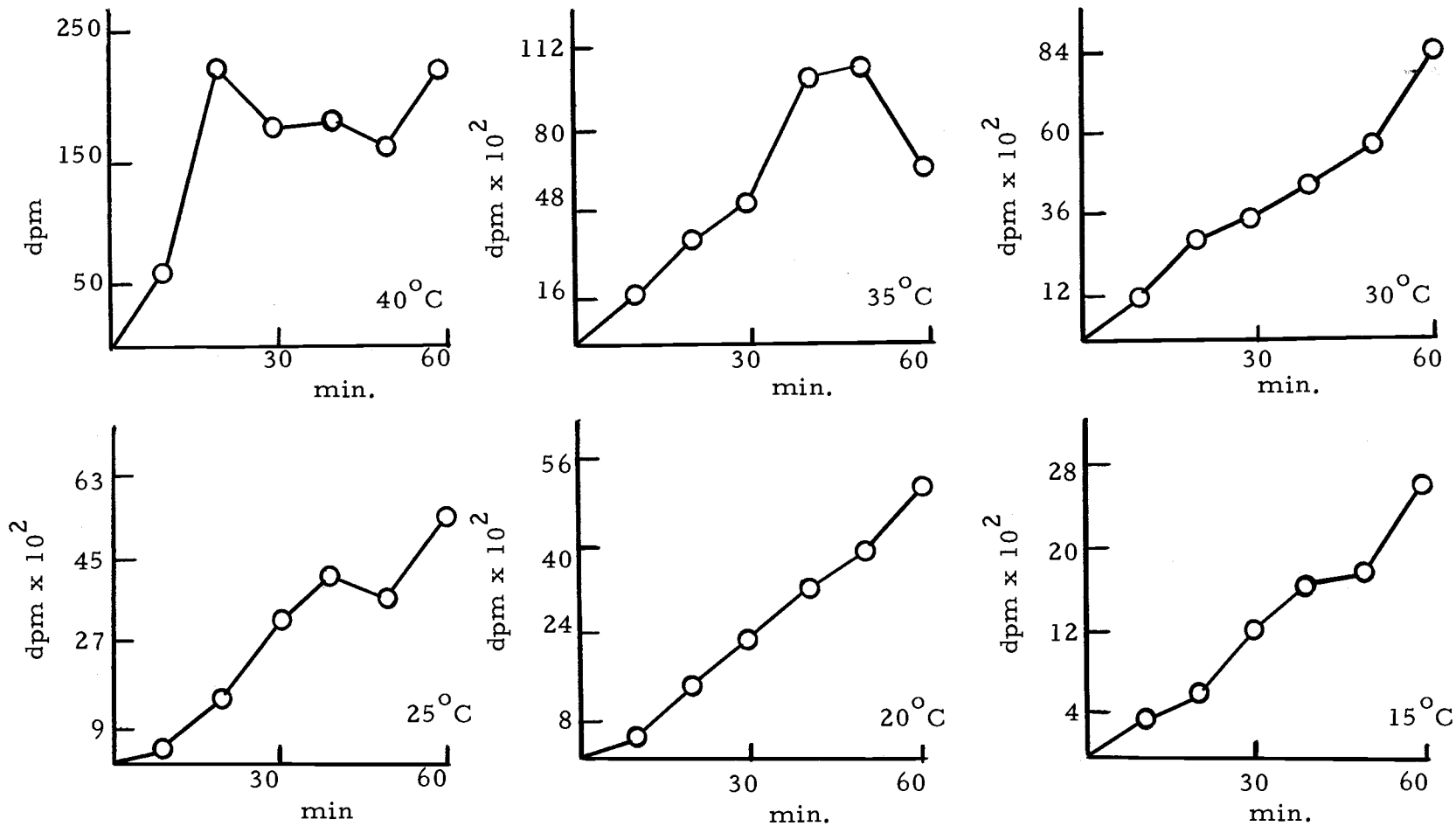

Figure 5. Cumulative ASMT activity over a one hour incubation period is plotted against time for six different incubation temperatures. Points are mean DPM values.

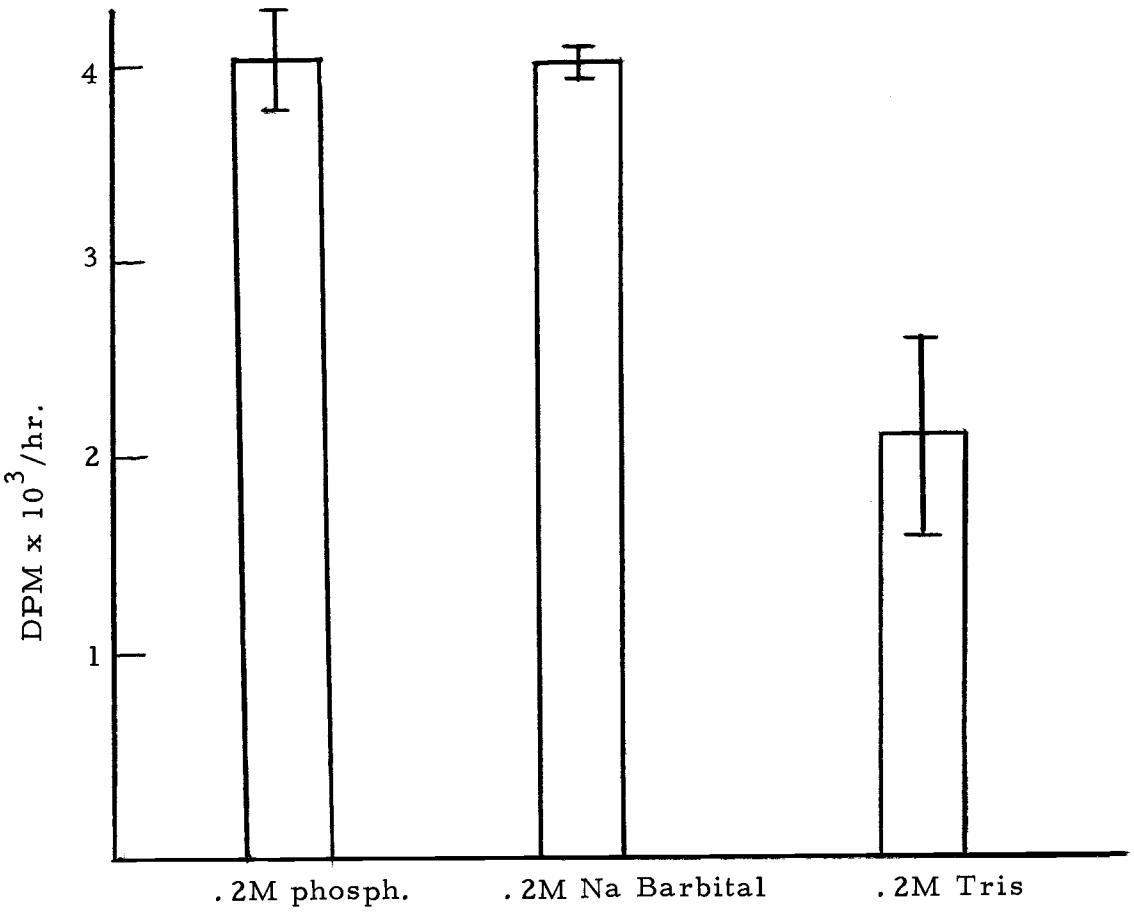

Figure 6. ASMT activity of a single homogenate after a one hour incubation is expressed as a function of three different buffer types. Columns represent mean values $\pm$ s.d.

Diurnal Studies

Mammalian studies indicate that there is a diurnal change in pineal ASMT activity (Axelrod, 1965), and this study ascertained that a similar fluctuation can be detected in steelhead pineals. In Figure 7, with activity expressed as a function of total pineal protein, a rhythmic pattern of activity with a low at 8:00 PM and a peak at 4:00 AM is attained. Statistical analysis reveals that these points are significantly different ($P < .05$).

In Figure 8 activity is expressed as a function of mean body weight and the pattern seen previously changes somewhat. Now a low occurs at noon and a peak is seen at 8:00 AM. Both 4:00 AM and 8:00 AM results are significantly greater ($P < .05$) than noon, 8:00 PM, and midnight activities. Another feature is the unusual spike at 4:00 PM, which makes that mean significantly greater ($P < .05$) than the mean at noon.

Pinealectomy Studies

The retinae of mammals and fish have been treated as secondary sites of melatonin synthesis. This study investigated the possibility that the pineal may play a regulatory role with regard to retinal melatonin production. Figures 9 and 10 deal with the effects of pinealectomy upon retinal ASMT levels. The

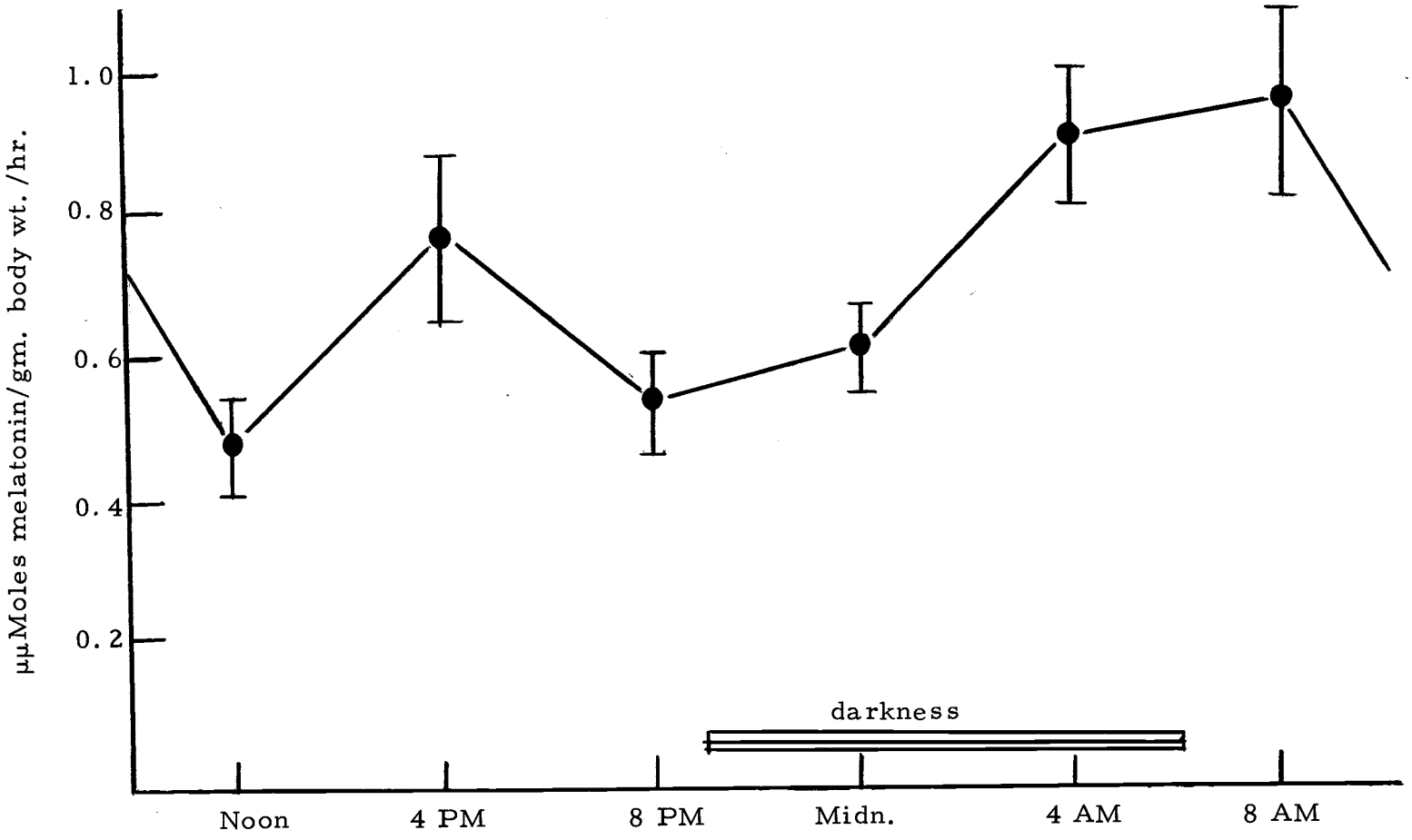

Figure 7. Melatonin production over a 24 hour period expressed as a function of mean body weight. Individual points represent mean values ± s.e.

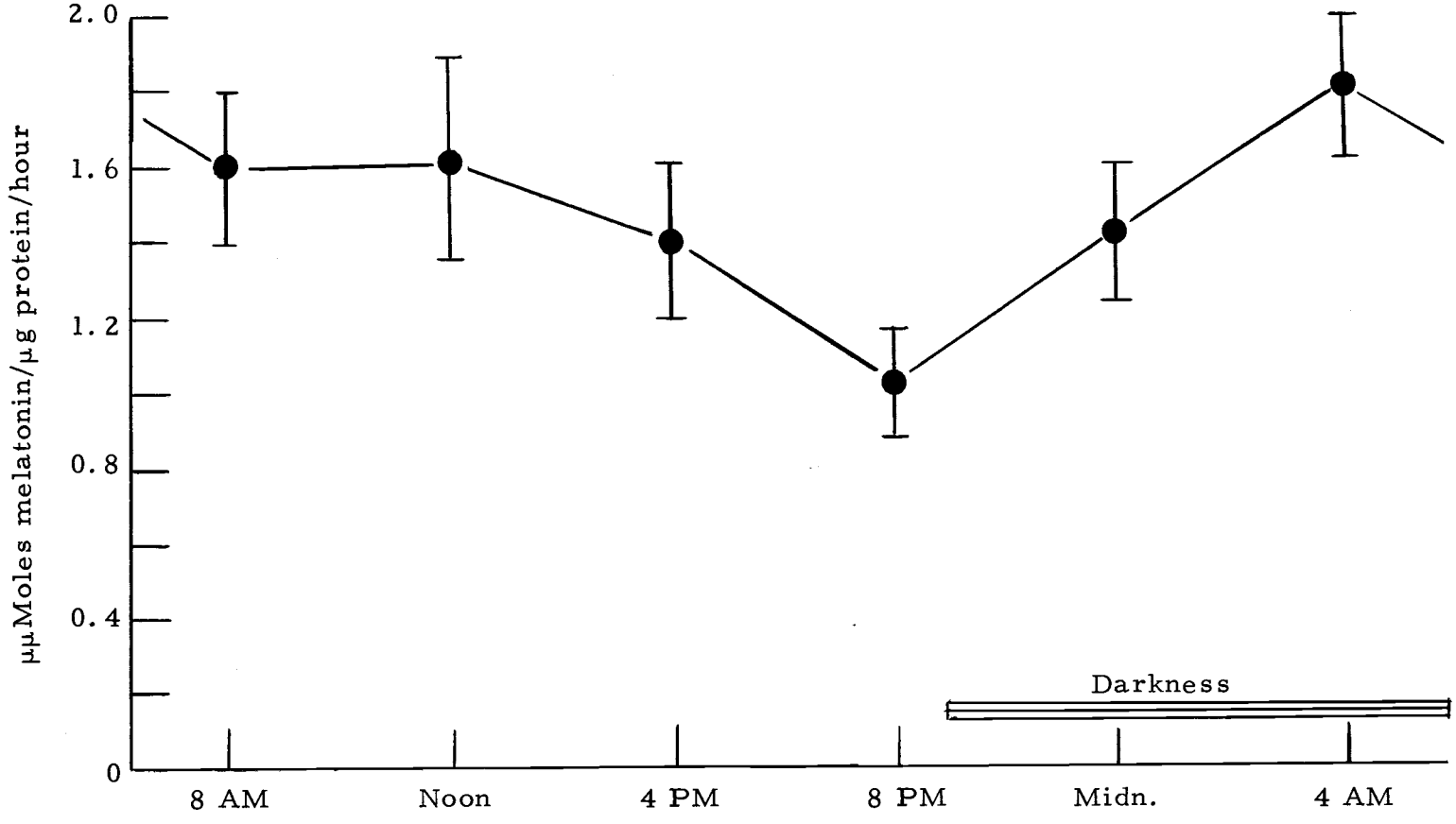

Figure 8. Melatonin production over a 24 hour period expressed as a function of sample protein content. Values are expressed as means ± s.e.

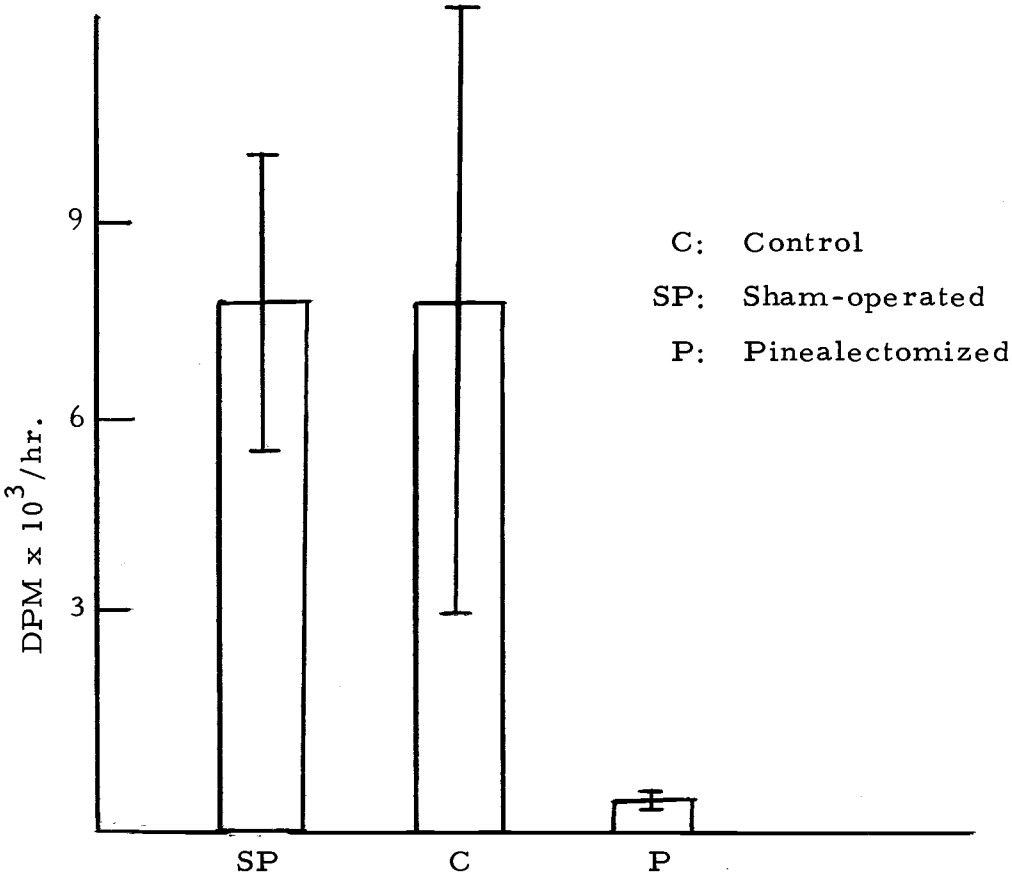

Figure 9. Pineal ASMT activity in DPM $\pm$ s.d. is represented by the columns for three different groups of fish

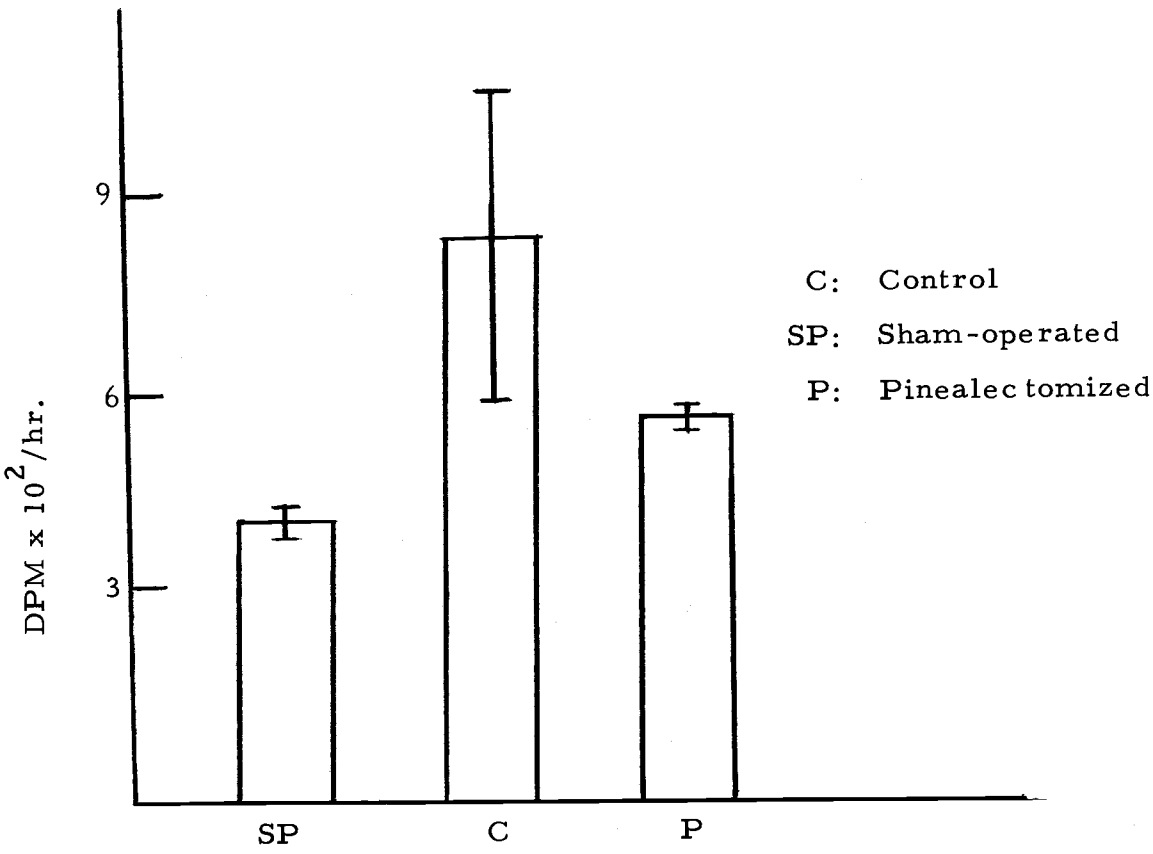

Figure 10. Retinal ASMT activity in DPM $\pm$ s. d. for three different groups of fish is represented by the three columns

study was unable to demonstrate statistically ($P < .05$) any difference in retinal ASMT levels following pinealectomy or sham-pinealectomy.

DISCUSSION

Before embarking upon any extensive study of trout ASMT activity it was felt that the assay parameters in current use should be thoroughly reviewed, since they closely resembled the parameters used in mammalian studies. Reports of species differences in the ASMT molecule (Wurtman, 1968), and the great physiological differences between fish and mammals increased the concern.

The results reported here show that no major parameter revisions are indicated, although a few minor modifications were adopted. The optimal pH of 7.9 is identical to the optimum reported for mammalian ASMT, which is in agreement with the comparative studies of Dewaide (1971). Of the buffer molarities studied, none was superior in terms of eliciting ASMT activity, however, the 0.2M incubation buffer showed considerably less variation and was consequently adopted for the assay. Temperature considerations were deemed very important since steelhead are poikilotherms and unable to tolerate 37°C, the incubation temperature employed in mammalian ASMT studies. Dewaide, working with the N-demethylating enzymes of rat and trout livers, reported striking differences in the response of these enzymes to temperature. He showed that optimal 10 minute activities occur at 42°C with the rat enzyme and at 25°C with trout enzyme, and that the trout enzyme

remained stable for 30 minutes at 25°C but showed an immediate loss of stability at 37°C. The results reported here show a similar pattern for the steelhead trout ASMT, with stability diminishing rapidly over a one hour preincubation period at temperatures above 30°C. Trout ASMT, however, appeared to be somewhat more stable than the N-demethylating enzyme, since the actual decline in enzymatic action did not occur for 30 minutes at 40°C and not until 40 minutes at 35°C. These results indicate that adequate activity as well as significantly greater stability can be achieved at 25°C as opposed to 37°C, and, since 25°C represents a temperature approaching more natural conditions for steelhead, it was adopted as the incubation temperature.

In conjunction with the parameter testing a study of the effects of freezing upon trout ASMT activity was conducted. Results show no significant change in enzymatic activity over a four day period within which all assays were completed.

Martini (1971) observed that animals normally survive pinealectomy and return to a normal state, which might suggest additional sites of synthesis of pineal products. ASMT has been located in the retina of certain fish (Quay, 1965), an observation which invites the speculation that a compensatory increase in ASMT activity might follow pinealectomy. The results reported here failed to corroborate this theory since no change in retinal activity

could be demonstrated. The possibility exists that the time allotted for post surgical recovery was insufficient, or that the assay procedure was inappropriate for retinal ASMT determinations.

The results of the diurnal rhythm studies when pineal ASMT activity is expressed as a function of total pineal protein show that a daily fluctuation is occurring in the juvenile steelhead trout. The zenith of this activity is 4:00 AM and the nadir at 8:00 PM, which, at the time of year this study was conducted, means peak activity occurs about five or six hours after onset of darkness, and the lowest activity occurs about one hour before the onset of darkness. These results are quite similar to results achieved by Axelrod (1965) using rats under controlled lighting conditions and strongly suggests a regulatory function for photoperiod with respect to steelhead trout ASMT activity. It is important to note that all assays were conducted under identical conditions and within a short period of time, indicating that the changes observed are due to the amount of enzyme present, a conclusion made also by Axelrod (1965) on the basis of experiments in which puromycin blocked the dark-induced rise in rat ASMT activity. Therefore, when referring to ASMT activity, it should be recognized that these are also references to ASMT levels.

The magnitude of change observed by Axelrod (1965) using

rat ASMT was from 1.6 to three fold, whereas the change reported here is very close to the lower limit of this range. Greater fluctuations in steelhead trout ASMT activity may be masked by the large variations encountered, which arise from a number of sources. Lighting undoubtedly contributed to the variation since all fish were held outdoors under natural lighting conditions, and consequently their respective photoperiods were not only changing constantly from day to day but also within the same day. Hopefully, some of this variation was eliminated by taking all samples within a four week period, two weeks before and two after the summer solstice. Another source of variation may also have been sex differences.

As mentioned earlier, there is good evidence that the ability of the steelhead trout pineal to produce melatonin can be affected by environmental lighting. Assuming this to be the case, it is interesting to speculate about some of the implications suggested earlier concerning pineal-endocrine relationships and migration.

The thyroid gland has for some time been implicated in the physiological changes that occur in migratory species of fish which create a "migratory disposition" (Hoar, 1963). One such change in diadromous fish is the change in water preference, i.e., from a saltwater preference to a freshwater preference, or conversely, from freshwater to saltwater. Baggerman (1963) has shown that pink salmon treated with thyroid inhibiting agents change preference

from saltwater to freshwater. In mammals, melatonin has been shown to be a thyroid inhibiting agent (Panda and Turner, 1968; Bashieri et al., 1963; De Prospo, De Martino, and McGuinness, 1968), and, if this property can be attributed to teleost melatonin, the pineal may play an important role in the timing of thyroid induced changes. A hypothetical sequence of events leading to the upstream migration of steelhead in winter might be initiated by the lengthening nights of fall, leading to an increased ASMT level, producing a rise in melatonin and a consequent depression of thyroid activity and eventually freshwater preference. Downstream migration of juveniles could be explained in similar terms by opposite effects induced by the increasing daylight of spring and early summer. Interestingly, Eales (1936) has shown that thyroid activity is greater in juvenile steelhead trout under long term photoperiods if the fish are of migrant age.

Another important physiological requirement necessary for the migration of diadromous fish is the adjustment of their osmoregulatory systems before moving between fresh and saltwater. Prolactin is considered the hormone which facilitates these adjustments, since electrolyte imbalances in fish that are suddenly moved from saltwater to freshwater can be prevented by the injection of prolactin (Lam and Leatherland, 1969; Lam, 1968; Lam and Hoar, 1967). Similar to an injection of prolactin would be an increase in

the secretion of endogenous prolactin, an event which in mammals can be initiated by melatonin (Kamberi, Mical, and Parter, 1971; Relkin, 1972). If steelhead melatonin has this property, then it might be hypothesized that a decrease in light in fall may cause a rise in ASMT levels with a subsequent increase in melatonin and a corresponding increase in plasma prolactin. The prolactin would then facilitate the osmoregulatory adaptations necessary for ascending the streams. The opposite could be envisioned for the juveniles, for as they mature in early spring and summer, their ability to osmoregulate in freshwater may be impaired by a decrease in plasma prolactin, due to the lengthening day, necessitating a seaward movement.

Hafeez and Quay (1970) were unable to show a light response in rainbow trout ASMT levels, which is in conflict with the results reported here. Differences might possibly be explained on the basis of methodology, yet there is also the possibility that this is a species specific difference. Steelhead trout and rainbow trout are very closely related, yet the fact remains that the steelhead is anadromous while the rainbow is a permanent freshwater resident, which might mean the steelhead pineal is more responsive to environmental lighting, contributing to its migratory behavior.

This study shows only that there is a diurnal change in ASMT levels in the pineal gland of juvenile steelhead trout. The regulator of this rhythm was not investigated, although it is tempting to speculate that environmental lighting has a role to play.

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