

## Comment on "Mechanical Resonance Spectra in Human Cancellous Bone"

**Abstract.** *The existence of two series of mechanical resonance frequencies in human cancellous bone is apparently supported by other results. This effect may be a microstrain phenomenon since all the experiments involved strains below  $10^{-4}$  at the frequencies of interest.*

In the preceding report, Pugh *et al.* (1) describe the observation of sharply frequency dependent reductions in the dynamic mechanical modulus of human cancellous bone. These are identified as consisting of two spectral series, corresponding to the momentum wave mode spectra for atomic calcium and phosphorus, as predicted by the theory of Fitzgerald (2). This finding of Pugh *et al.* is apparently supported directly by previously unexplained results of Black and Korostoff (3) and Black (4) and indirectly by the data of Smith and Keiper (5) on human cortical bone.

In a study of eight specimens of human tibial cortical bone, tested at 37.5°C with the force parallel to the major fiber axis, I observed significant reductions in the dynamic mechanical modulus in six cases (4). The limiting frequencies between which the minima in the dynamic modulus occurred are given in Table 1. The frequencies I observed were more widely separated than those of Pugh *et al.*, but the data suggest that the resonance occurs at or near the phosphorus frequency  $\nu_1^P = 203$  hertz reported by Pugh *et al.* This is despite the fact the value of the dynamic modulus at 165.8 hertz varied from  $1.16 \times 10^6$  to  $1.69 \times 10^6$  pounds per square inch (psi) (1 psi  $\approx$  52 torr), about 15 times the value reported for cancellous bone. The maximum reduction in modulus was 57 percent, while the strain in this frequency region was between  $2 \times 10^{-4}$  and  $5 \times 10^{-4}$ . The introduction of a reference resonance precluded the observation of  $\nu_1^{Ca}$ , and  $\nu_2^{Ca}$  could not be observed because of frequency limitations on the linear variable displacement transducers.

Table 1. Frequencies on the unperturbed dynamic modulus curve bracketing minima observed for six specimens of human tibial cortical bone (4).

Samples (No.)	Frequency (hertz)	
	Lowest	Highest
2	165.8	206.2
1	165.8	223.6
2	187.2	223.6
1	206.2	239.8

In addition to the work of Jurist (6) cited by Pugh *et al.*, the earlier study by Smith and Keiper (5) seems to indicate the resonance phenomenon indirectly. In a study of the viscoelastic properties of bone, Smith and Keiper measured the dynamic modulus of human cortical bone in a direction parallel to the fiber and concluded that the elastic component of stiffness,  $E'$ , for a strain of  $10^{-6}$  was independent of frequency between 500 and 3500 hertz. However, in attempting to measure the viscous component,  $E''$ , they observed irregularities which they described as "scatter." In retrospect, I believe that they accidentally observed some of the  $E''$  peaks predicted by

Pugh *et al.*, probably those at 812 hertz ( $\nu_2^P$ ) and 1827 hertz ( $\nu_3^P$ ) on the basis of their relative magnitudes.

In conclusion, I believe that the observations reported by Pugh *et al.* are soundly grounded on previous work and are unlikely to be experimental artifacts. Whether these resonances are the momentum wave effects predicted by the theory of Fitzgerald must remain an open question until experiments can be performed on model lamellar systems of rigidly controlled purity and geometry.

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## Pineal Enzymes: Regulation of Avian Melatonin Synthesis

**Abstract.** *Groups of 8-week-old chickens were killed at six time points in their light-dark cycle, and pineal glands were removed and assayed individually for N-acetyltransferase activity, hydroxyindole-O-methyltransferase activity, and melatonin content. The tenfold nocturnal rise in melatonin was phased identically with the 27-fold increase in N-acetyltransferase activity. The relatively small changes (20 percent) in hydroxyindole-O-methyltransferase activity did not appear important in causing the large changes in melatonin. The phase of the rhythms of N-acetyltransferase activity and melatonin content in chickens relative to the phase of the light-dark cycle was qualitatively similar to that of rats. In contrast, the sleep-wake cycle of chickens is about 180° out of phase with that of rats.*

Melatonin is synthesized from serotonin by *N*-acetylation, catalyzed by serotonin *N*-acetyltransferase, and *O*-methylation, catalyzed by hydroxyindole-*O*-methyltransferase (HIOMT) (1). Daily rhythms in pineal melatonin content, serotonin content, *N*-acetyltransferase activity, and HIOMT activity have been studied in birds; but it has not been clear which enzyme, if either, regulates the rhythms in pineal melatonin and serotonin (2-5). Klein and Weller (6) presented evidence that the melatonin and serotonin rhythms in the rat pineal gland are regulated by *N*-acetyltransferase activity. We examined the daily rhythms of pineal *N*-acetyltransferase activity, HIOMT activity, and melatonin content in an experiment in which all three determinations were made on homogenates of each pineal gland. In this experi-

ment, *N*-acetyltransferase activity appeared to be a major regulatory factor for the avian melatonin rhythm.

We used 8-week-old chickens (White Leghorn cockerels, *Gallus domesticus*) that had been kept from the day after hatching in a light-dark cycle of 12 hours of light followed by 12 hours of dark (LD 12:12). Groups of six chickens were killed at six time points in this cycle, and the pineal glands were quickly dissected out and frozen. The glands were later thawed and individually homogenized; HIOMT activity, *N*-acetyltransferase activity, and melatonin content were measured in samples of the homogenate (7). In a separate series, body weights and pineal weights of eight chickens and eight rats were determined (8) to provide a basis for quantitative comparisons of the biochemical data from the two species.

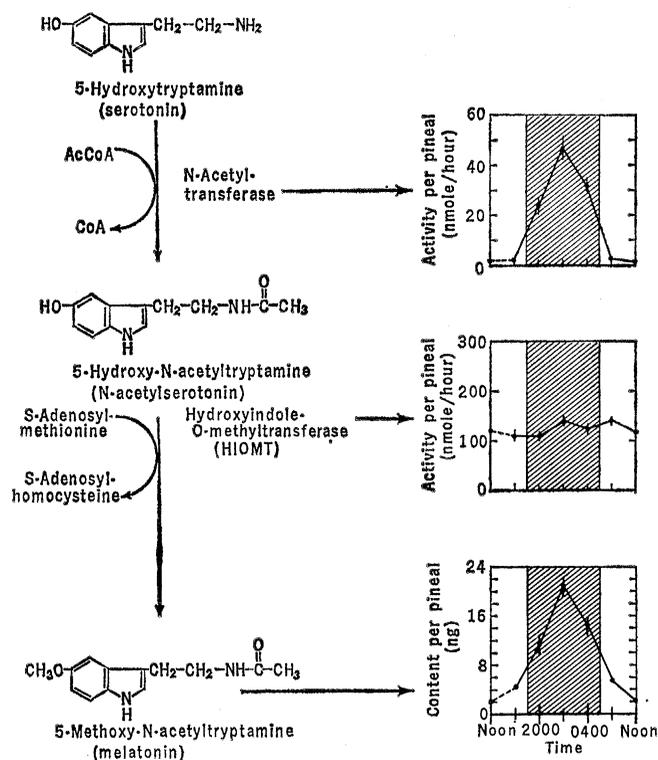


Fig. 1. The accepted pathway for melatonin synthesis in the pineal gland (left) and graphs of *N*-acetyltransferase activity, HIOMT activity, and melatonin content in chicken pineal glands (right). The standard errors are indicated where they exceed the size of the symbol. The curves consist only of six points each, and the connecting lines may not truly delineate the shape of the light cycle. The hatched area represents the dark portion of the light cycle. *N*-Acetyltransferase activity was measured (7) with one-tenth of a homogenized chicken pineal gland. Activity of HIOMT was measured by the method of Pelham and Ralph (5) with slight modifications: The pineal homogenate added was one-twentieth of a pineal gland in 5  $\mu$ l of 0.1*M* phosphate buffer, pH 6.8. The final reaction mixture contained 255  $\mu$ l at pH 7.9. All samples were first incubated for 10 minutes without substrates. The reaction was stopped with 2 ml of 0.45*M* borate buffer, pH 9.95. Melatonin was extracted into 10 ml of chloroform; the extract was washed once with borate and once with 1*N* HCl, and a 5-ml portion was evaporated and the residue was counted. Melatonin was measured by the tadpole bioassay (7). One-tenth of a chicken pineal gland in 10  $\mu$ l was diluted with 1 ml of deionized water and frozen for shipment to Pittsburgh where the samples (including buffer blanks) were thawed at room temperature 1 hour before assay and, if necessary, diluted with distilled, deionized water to bring the sample into the desired concentration range. All samples were coded, and the melanophore index of the tadpoles was determined independently by two people. The specificity of the melatonin bioassay has been discussed (7). No points of these curves represent zero or unmeasurable enzyme activity or melatonin content; all pineal glands had quantities well within the range of the assays; AcCoA, acetyl coenzyme A; CoA, coenzyme A.

*N*-Acetyltransferase activity varied by a factor of 27 in the 24-hour cycle, and melatonin content varied by a factor of 10 (Fig. 1). Peak activity occurred in the dark, when chickens are normally inactive. Values for HIOMT activity fluctuated by only 20 percent, and the high values did not all occur at night; indeed, the high HIOMT value at 0800 occurred when melatonin content had already dropped significantly. In order to evaluate statistically the relation between the two enzyme activities and melatonin content, we calculated correlation coefficients between the values obtained at the six time points for individual animals. A positive correlation between *N*-acetyltransferase activity and melatonin content ( $r = .97$ ) was highly significant ( $P \ll .001$ ), as evaluated by use of Fisher's *z*-transformation. A significant correlation was found between HIOMT and *N*-acetyltransferase activities ( $r = .38$ ;  $P < .05$ ); that between HIOMT activity and melatonin content was insignificant ( $r = .22$ ) (9).

Our data for chickens and rats (midnight values) indicate that the chicken has a much larger enzymatic capacity for the synthesis of melatonin (9). When data are expressed per gram of wet pineal, chickens have 7 times the *N*-acetyltransferase activity of rats, 600 times the HIOMT activity, and 1.3 times the amount of melatonin. When body weight rather than pineal size is

considered, chickens have 4 times the *N*-acetyltransferase activity of rats and more than 360 times the HIOMT activity, but the two species have similar quantities of melatonin in their pineal glands. The changes in HIOMT activity in constant darkness are in opposite directions in day-active chickens compared to night-active rats (9). However, melatonin content and *N*-acetyltransferase activity have similar daily rhythms in both animals; that is, these values are high at night and low in the day for both species. The phase of these rhythms is not determined by the nocturnal or diurnal life-style of the organisms, but, rather, is linked to the environmental lighting and the activity state normally consonant with that lighting. That is, high *N*-acetyltransferase activity and melatonin content are associated with darkness and locomotor activity in rats, but with darkness and locomotor inactivity in chickens. If this constitutes a system for keeping track of environmental lighting, it may be especially important to reproduction, which is controlled by the lengths of the daily dark and light periods in many species.

From the data presented here, it appears that cyclic changes in pineal melatonin content in chickens are regulated by production of *N*-acetylserotonin at the *N*-acetyltransferase step. Thus, this regulatory mechanism seems to exist in two classes of verte-

brates, mammals (6) and birds. The rhythm in serum melatonin concentration in chickens is in phase with the rhythm in pineal melatonin content, and is abolished by pinealectomy (10). These observations indicate that in birds, pineal melatonin release is probably a passive process determined by the rate of melatonin production. Melatonin release in mammals has not been studied *in vivo*; however, in cultures of rat pineals, the regulation of melatonin appearance in the culture medium has been linked with the regulation of melatonin production at the *N*-acetyltransferase step (11).

Although HIOMT does not appear to be crucial to the rhythmicity of melatonin production, the enzyme does exhibit reproducible changes in activity when animals are subjected to periods of constant light or darkness for 5 days or longer (3, 12). It is possible that HIOMT activity exhibits tonic responses to environmental lighting that require several days to develop, whereas *N*-acetyltransferase activity is more rapidly responsive to environmental lighting on a daily rhythmic basis. Barfuss and Ellis (13) reported an annual cycle in HIOMT activity that is the mirror image of the testicular cycle in sparrows. This supports the seasonal role for HIOMT in regulation of melatonin synthesis in birds, as suggested by Bäckström *et al.* (4).

Physiological experiments show a

possible relation between the pineal gland and circadian rhythms in two ways: (i) The pineal gland is necessary in sparrows for the persistence of the normal endogenous rhythms of body temperature and locomotor activity, and both sleep and body temperature changes are produced by injection of the putative pineal hormone, melatonin (14). (ii) The hamster pineal gland has been linked to reproductive phenomena in ways that involve day length and circadian rhythms (15). It is not yet possible, however, to fully relate the biochemical findings to the physiological studies. The circadian rhythm of locomotor activity of some but not all vertebrates (16) may be dependent on a pineal melatonin rhythm controlled by *N*-acetyltransferase activity.

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random groups of six at 4-hour intervals (0400, 0800, 1200, 1600, 2000, and 2400) during a single 24-hour period. For decapitation in the dark period, animals were exposed for 4 seconds to dim light (2 lu/m<sup>2</sup>) from a 7.5-watt red lamp (General Electric). Following decapitation, the heads were placed immediately on ice, and the pineal glands were quickly dissected out and individually frozen on Dry Ice in coded plastic vials. The vials were shipped on Dry Ice the following day to Bethesda, where the enzyme assays were performed without prior knowledge of experimental details. After the enzyme assays, samples of the pineal homogenates were diluted and returned to Pittsburgh on Dry Ice, where they were assayed for melatonin within 3 days of decapitation. Assay methods were as follows. *N*-Acetyltransferase: N. Ellison, J. Weller, D. C. Klein, *J. Neurochem.* **19**, 1335 (1972). HIOMT: Pelham and Ralph (5). Melatonin: C. L. Ralph and H. J. Lynch, *Gen. Comp. Endocrinol.* **15**, 334 (1970); H. J. Lynch, in *Workshop on the Pineal Gland*, David Klein, Ed. (Raven, New York, in press).

8. Eight normal rats (average weight, 178 g) and eight normal chickens (average weight, 695 g) were decapitated. The pineal glands were dissected out, cleaned of adhering connective tissue and extracapsular vascular tissue, blotted, and weighed in the wet state. The stalks were not included. For comparison of rat and chicken enzyme values, a nocturnal *N*-acetyltransferase activity value of 3 nmole per pineal per hour was used.
9. The assays for melatonin and *N*-acetyltransferase activity were identical to those used for rats; therefore, the results obtained in chickens and rats are comparable. However, because the optimum assay conditions for rat and chicken HIOMT differ greatly, the HIOMT data are not strictly comparable. We directly compared chicken and rat pineal HIOMT activities by using the rat assay described below. In this experiment, the chicken activity was 34 times the rat activity, but the conditions were far from optimal for measuring chicken HIOMT activity. The rat microassay was modified from the method of J. Axelrod and H. Weissbach [*J. Biol. Chem.* **236**, 212 (1961)]. The reaction mixture (final volume, 20  $\mu$ l) contained 0.1 mM *S*-[<sup>14</sup>C]adenosylmethionine (52 mc/mole) and 0.02 mM *N*-acetylserotonin. The final pH was 7.4. With both types of HIOMT assay we confirmed that some concentrations of *N*-

acetylserotonin inhibit HIOMT activity in birds, as reported by B. Alexander, A. J. Dowd, A. Wolfson [*Neuroendocrinology* **6**, 236 (1970); (5)].

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7. Thirty-six White Leghorn cockerels were obtained as day-old chicks from Thomas Mack and Sons of West Sunbury, Pennsylvania, and raised in Pittsburgh under a standard 24-hour photoperiod (lights on from 0600 to 1800 E.S.T.) with free access to food and water. The birds were housed in the topmost tiers of two adjacent cages with wire mesh covers (G.F.Q. Mfg. Co., Savannah, Georgia), each situated directly beneath a fluorescent ceiling lamp providing "cool white" illumination of approximately equivalent intensity (1380 to 1480 lu/m<sup>2</sup> measured at the cage tops by a Weston photometer, model 756). When 8 weeks old, the birds were decapitated in

## Energy Expenditure in Animal Locomotion

**Abstract.** *A wide variety of data on the energy expended by animals in running, flying, or swimming can be accounted for by the simple hypothesis that all animals require the same quantity of energy to carry a unit of their own body mass one "step." For running locomotion this is approximately  $3 \times 10^{-4}$  calorie per gram per step.*

Schmidt-Nielsen (1) has reviewed a remarkable range of data on the energy which animals must consume to achieve locomotion. He defines a speed-independent consumption rate, which I will call the specific energy cost, *C*, and measure in units (2) of calories per gram per centimeter. This is the energy price an animal must pay to move 1 g of its own weight 1 cm. He presented the data in the form of logarithmic plots of *C* as a function of body mass (3). For as wide a range as six orders of magnitude in mass, the resulting plots are straight lines. Data for running, flying, and swimming are compared and show that swimming is more energeti-

cally efficient than flying, which is, in turn, more efficient than running.

A most striking feature of these data, especially when presented together as in figure 4 of (1), is the similarity of the slopes of the fitted lines for all three modes of locomotion. In discussing the data on running, Schmidt-Nielsen notes that they are fit by a regression line with slope  $-0.40$ , but he tenders no explanation.

I take a less statistical and more intuitive view of these plots. Using the eye as a guide, I assert that the data are fairly represented by the statement that the specific energy cost, *C*, is proportional to the inverse cube root (the