Circadian Rhythms of Melatonin Release from Chicken Pineal *In Vitro*: Modified Melatonin Radioimmunoassay (43320)

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Abstract. **An improved and simplified radioimmunoassay for measuring pineal, serum, and *in vitro* cultured medium melatonin is described. Using 2-[^{125}I]iodomelatonin as radiolabeled ligand and a polyclonal rabbit antimelatonin antiserum, melatonin concentrations were determined in all three types of samples by a 2-day direct equilibrium double-antibody assay method without prior extraction. Serial dilutions of pineal homogenates, serum, and cultured medium all gave parallel displacement curves. Cross-reactivity of the antisera with other indoles was negligible. Intraassay coefficients of variation (n = 3) were 5.09, 3.32, and 5.05% at 7.81, 62.5, and 500 pg/tube, respectively, and the interassay coefficients of variation (n = 20) were 12.18% at 62.5 pg/tube. A characteristic diurnal rhythm of melatonin was observed using this direct assay for measuring daytime and nighttime chicken pineal and serum samples. An *in vitro* incubation of chicken pineal glands with a lighting cycle of 12-hr light:12-hr dark showed that the diurnal rhythm of melatonin secretion into the cultured medium was maintained. The direct assay method described in this report for measuring chicken melatonin using 2-[^{125}I]iodomelatonin as radiolabeled ligand coupled with the *in vitro* cultured chicken pineal gland clearly offers great potential for studying the chicken pineal circadian oscillator and its underlying mechanism.** [P.S.E.B.M. 1991, Vol 198]

Study of pineal physiology depends heavily on the availability of specific and sensitive assays to measure pineal indoles present in different biologic samples. Currently, pineal indoleamines can be determined by radioimmunoassay (RIA), gas chromatography-mass spectrometry, and high-pressure liquid chromatography methods (1); however, most of these assay methods require time-consuming extractions with organic solvents before analysis. A melatonin RIA with no extraction step has been developed (1, 2), and this direct assay has been used to measure melatonin in human, ovine, rat, and marsupial plasma and human saliva. Because the direct assay involves using radioiodinated melatonin rather than tritiated melatonin as a tracer, costly and cumbersome scintillation counting can be avoided.

It is well documented that the chicken pineal gland maintains its circadian rhythm when cultured in *in vitro* organ culture (3–6). These cultured chicken pineal glands have photoreceptive capacity and remain photosensitive in culture; the physiologic activity can also be entrained by light cycles. Most of these studies measured the activity of the enzyme serotonin *N*-acetyltransferase (NAT) as an index of the pineal function (3–5). Serotonin *N*-acetyltransferase converts serotonin to melatonin. Using NAT activity as an end point, Binkley *et al.* (3), Deguchi (4), Kasal *et al.* (5), and Wainwright and Wainwright (7) reported an increase and decrease of NAT activity in isolated pineals. Kasal *et al.* (5) showed that the rhythm persists for two cycles in constant darkness. Using a superfusion *in vitro* method, Takahashi *et al.* (6) showed that circadian rhythms of melatonin release were maintained for as long as 5 days.

This article describes an improved, simplified, direct RIA for measuring melatonin in chicken pineal serum and cultured medium. Using this RIA, the release of melatonin from chicken pineal glands cultured *in vitro* in a static and a superfused incubation system was successfully measured.

Materials and Methods

Animals. Unsexed broiler and layer chickens (aged 4 to 6 weeks) were housed in a temperature-controlled

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(26°C) room with lights on between 0800 and 2000 hr. All birds were fed commercial chicken diet and water *ad libitum*. Pineal glands used for the *in vitro* studies were all obtained from broiler chickens. Pineal glands and serum samples for melatonin assay validation were obtained from layer chickens.

Chemicals and Reagents. BGJb media, HEPES buffer, L-ascorbic acid, melatonin, and its various indolic analogues were obtained from Sigma Chemical (St. Louis, MO). Fetal bovine serum was purchased from Hyclone (Logan, UT). Gentamicin sulfate was purchased from Grand Island Biological Co. (Grand Island, NY). [^{125}I]-Melatonin (NEX-236, 50 μCi) was obtained from Dupont (New England Nuclear, Boston, MA). Melatonin antiserum (CIR 103) was obtained from CIDtech Research, Inc. (Ontario, Canada).

Culture Medium. Culture medium was prepared by sterile techniques. Each liter of BGJb contained fetal bovine serum (10%), HEPES buffer (10 m*M*), L-ascorbic acid (2 m*M*), and gentamicin sulfate (500 mg). Media pH was adjusted to 7.6.

Preparation of Static Culture. Chickens were sacrificed between 9 and 11 AM and pineal glands were surgically removed from decapitated birds and placed in culture medium on ice. Before glands were cultured, they were equilibrated by washing three times in 40°C culture medium. One, two, three, or four glands were placed in a Falcon organ culture plate fitted with a presterilized stainless steel screen. Each culture plate received 2.0 ml of 40°C culture medium and was placed in a 40°C incubator supplemented with a 95% O_2:5% CO_2 gas mix. An internally mounted 30-W fluorescent lamp was timed to maintain a 12:12-hr light:dark cycle inside the cabinet. Glands were allowed to incubate overnight, and medium was changed at 4-hr intervals thereafter. Dark-cycle samples were collected under red light. Medium samples from each plate were collected and stored at −20°C until melatonin RIA was performed.

Preparation of Perfusion System. Medium was prepared similar to the method described for the static culture. After washing, 10 glands were placed in a sterilized glass 1-ml tuberculin syringe used as a perfusion chamber. Groups of three or four glands were separated by presterilized sections of 500-μm Spectra/Mesh (Spectrum, Los Angeles, CA). The perfusion chamber was placed in the 40°C incubator on the same light:dark cycle as static cultures. Medium was kept in a 40°C water bath. Flow rate (4 ml/hr) was maintained for the duration of each experiment. Medium was collected by a fractionator set to collect 4-ml samples. Samples were collected and kept at −20°C until melatonin RIA was performed.

Melatonin RIA. Melatonin concentrations were determined by a variation of a 2-day double-antibody RIA procedure previously described by Leung et al. (8) for chicken growth hormone. All samples and standard were assayed at a final volume of 100 μl; 100 μl of a 1/6000 dilution of the primary antibody were used, and the assay was allowed to equilibrate for 48 hr at 4°C. After 48 hr of incubation, 100 μl of a 1/50 dilution of second antibody (sheep anti-rabbit γ-globulin; Cappel)

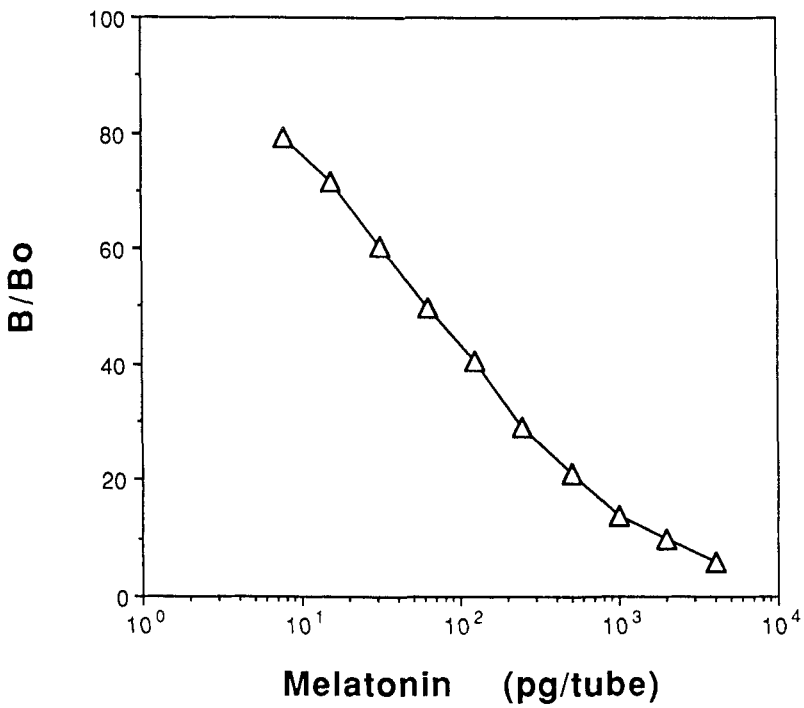

Figure 1. Representation of melatonin radioimmunoassay standard using double-antibody procedure.

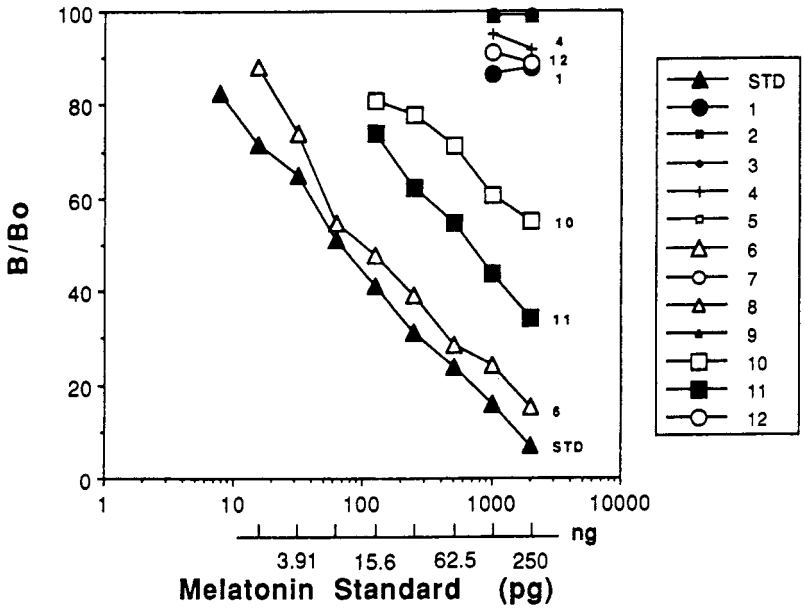

Figure 2. Displacement curves obtained from various indolic analogs. STD, melatonin standard; 1, 5-methoxytryptamine; 2, hydroxytryptamine HCl; 3, 5-hydroxindole 3-acetic acid; 4, 5-methoxytryptophol; 5, *N*-acetyl-L-tryptophan; 6, 6-hydroxy-melatonin; 7, 5-hydroxyindole 3-acetic acid (free acid); 8, 5-methoxyindole 3-acetic acid; 9, 5-methoxy-DL-tryptophan; 10, 5-hydroxytryptamine; 11, *N*-acetyl 5-hydroxytryptamine; 12, *N*-acetyl-D-tryptophan.

were added with 100 μl of 20% polyethylene glycol. The assay is allowed to equilibrate for 2 hr at 4°C. One milliliter of cold water was added to the reaction mixture and the samples were then centrifuged at 3500*g* (4°C) for 10 min. The supernatant was decanted and the precipitate was counted in a Micromedic automatic gamma counter (Horsham, PA). Samples from a given experiment were assayed in a single RIA. The standard was *N*-acetyl-5-methoxytryptamine (melatonin; Sigma).

Results

Radioimmunoassay Validation. A typical standard displacement curve using 2-[^{125}I]iodomelatonin as a radiolabeled ligand and a polyclonal rabbit antimelatonin antiserum is shown in Figure 1. ED_{50} of the assay is 66.81 ± 2.45 pg/tube (n = 23 assays). Intraassay coefficient of variation (CV) (n = 3) were 5.09% at 7.81 pg, 3.32% at 62.5 pg, and 5.05% at 500 pg. Interassay CV (n = 20) was 12.18% at 62.5 pg.

Cross-reactivity of various indolic analogs to displace bound [^{125}I]-melatonin from the antiserum was negligible (Fig. 2). The only measurable cross-reactivity expressed for the quantity of melatonin necessary to displace 50% of bound [^{125}I]-melatonin was 0.47% for 6-hydroxymelatonin, 0.09% for *N*-acetyl-5-hydroxytryptamine, and 0.03% for *N*-acetyltryptamine. Cross-reactivity of the other nine indolic analogues tested was <0.01% (Table I).

Serial dilutions of chicken pineal homogenates, serum, and incubation medium samples all showed parallelism to the melatonin standard (Fig. 3). Analytical recovery of melatonin was performed by adding melatonin standard to incubation culture medium (Fig. 4). Melatonin added to culture medium at 25, 50, 100, and 200 ng/ml was quantitatively recovered. The linear regression is $y = 0.98x - 2.85$; $r = 0.99$; y intercept, −2.85 pg. The endogenous concentration of melatonin in the incubation culture medium is below the minimal detection of the assay. Chicken pineal and serum concentrations showed a circadian rhythm, indicated by a 9-fold and 3-fold increase in the nighttime peak versus daytime minimal pineal and serum concentration. Daytime versus nighttime melatonin values were 7.27 ± 1.56 (n = 19) vs 61.88 ± 11.92 (n = 10) ng/gland and

Table I. Cross-Reactivity of Various Indolic Analogues as Determined by RIA Using Double-Antibody Procedure and Radioiodinated Melatonin as Tracer

Compound	Cross-reactivity[a] (%)
Melatonin	100.00
6-Hydroxy-melatonin	0.47
N-Acetyl-5-hydroxytryptamine	0.09
5-Hydroxytryptamine	0.03
5-Methoxytryptamine	<0.001
Hydroxytryptamine HCl	<0.001
5-Hydroxindole-3-acetic acid	<0.001
5-Methoxytryptophol	<0.001
N-Acetyl-L-tryptophan	<0.001
5-Hydroxyindole 3-acetic acid (free acid)	<0.001
5-Methoxy-DL-tryptophan	<0.001
N-Acetyl-D-tryptophan	<0.001

[a] Fifty percent displacement.

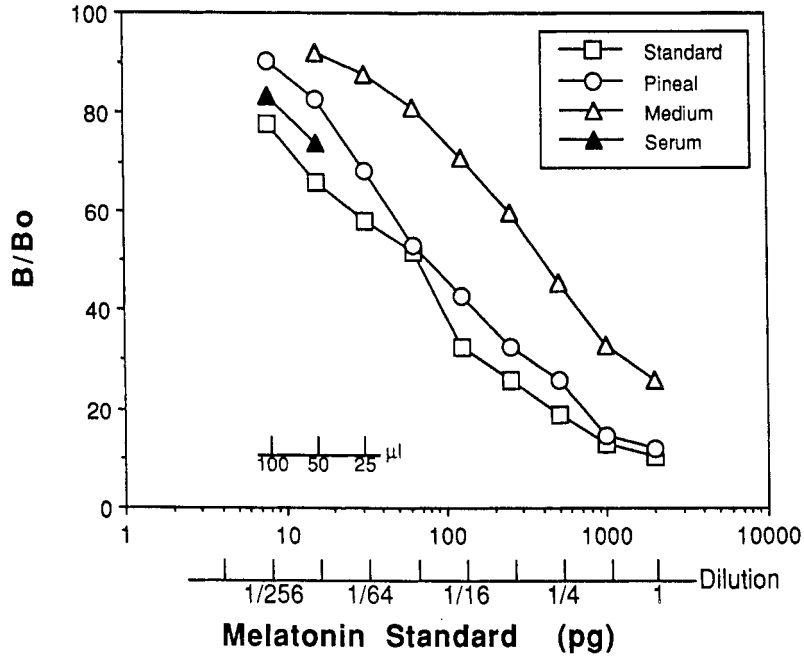

Figure 3. Displacement curves for chicken pineal, serum, and incubation medium samples in melatonin radioimmunoassay.

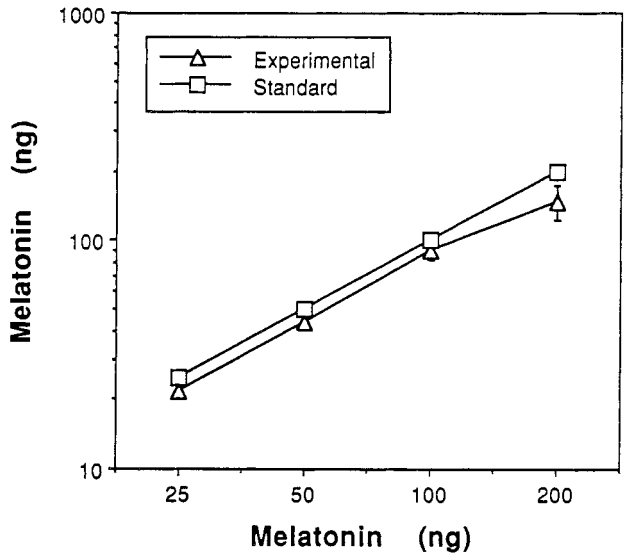

Figure 4. Analytical analysis of exogenous melatonin recovery measured in melatonin radioimmunoassay.

240.98 ± 25.34 (n = 24) vs 796.18 ± 59.26 (n = 23) pg/ml of serum.

***In Vitro* Studies.** In the static pineal organ incubation culture system, pineal glands released melatonin into the incubation medium rhythmically (Fig. 5). The rhythmicity of the melatonin concentration in the incubation medium followed the 12:12-hr light:dark cycle, reaching peak levels during dark and minimal levels during light periods. The amplitude of the melatonin is most significant during the second and third light:dark cycle. The diminished melatonin peak on the first light:dark cycle may be due to the disruption of the light phase when the pineal glands were kept on ice inside a protected cooler with no exposure to light during the 4- to 5-hr transit to the laboratory. The amplitude of melatonin nighttime peaks released by one, two, and three pineal glands in culture seemed to be stable for the second and third light:dark cycle, but the amplitude of melatonin nighttime peaks released by four pineal glands in culture was different. There is a significant decrease in the amplitude peak for the second and third nighttime peaks, ~800 versus ~200 ng/ml. Considering the second light:dark cycle melatonin values in this static culture system, on average a single pineal produced ~1 to 2 ng/hr during the day and ~20 to 25 ng/hr at night. The total amount of melatonin produced averaged ~200 ng in 24 hr.

In the superfused flow-through pineal organ culture, chicken pineal glands also maintained a circadian rhythm of melatonin release into the cultured medium following the 12:12-hr light:dark cycle (Fig. 6). Except for the first light:dark cycle, the general pattern of the rhythm is remarkably stable and uniform. Superfusate melatonin concentration reached minimal levels during the light and peak levels during the dark cycle. In almost every case, the increase in superfusate melatonin concentration preceded the lights-off transition and the decrease of superfusate melatonin concentration preceded the lights-on change. The decreased amplitude of melatonin during the first light:dark cycle again may be due to a light phase interception during transit. In this superfusion system, excluding the first light:dark cycle values, on average a single pineal produced ~1 to 2 ng/hr during the day and ~22 to 40 ng/hr at night.

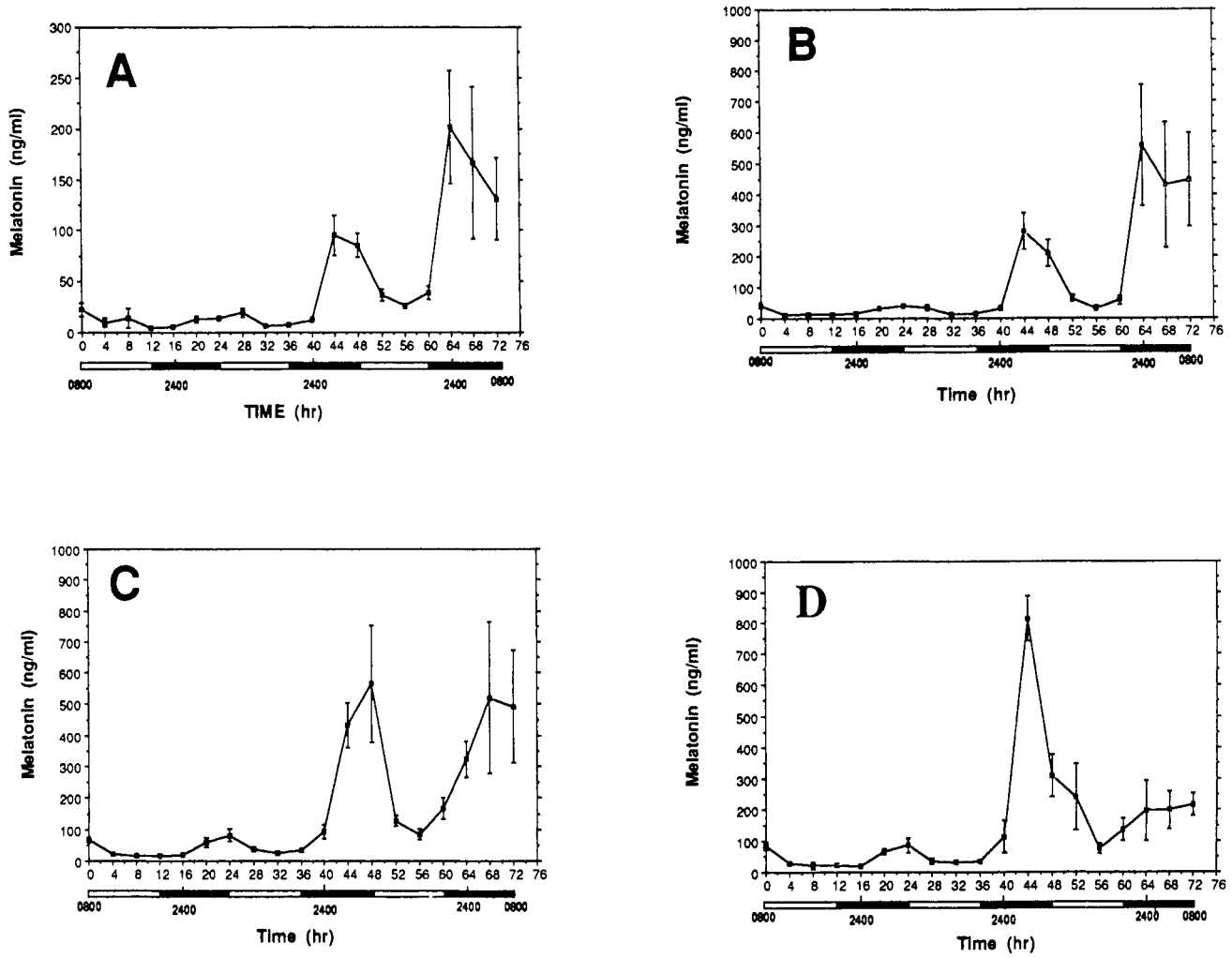

Figure 5. Circadian rhythm of melatonin released from single (A), two (B), three (C), and four (D) pineal glands cultured in static *in vitro* system. Melatonin concentrations are presented as mean ± SE (n = 4–6).

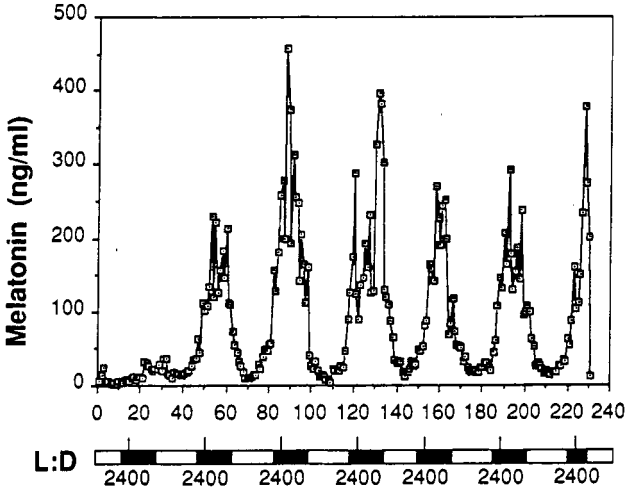

Figure 6. Circadian rhythm of melatonin released from chicken pineal glands in superfusion *in vitro* cultured system.

The total amount of melatonin produced averaged ~200 ng in a 24-hr period.

In both static and superfusion system experiments, the light:dark cycle was continued *in vitro* with the same phase as the previous *in vivo* light cycle.

Discussion

Most of the RIA developed for melatonin use tritiated melatonin as the radiolabeled ligand. The use of ^{125}I as radiolabeled tracer was first reported by Rollag and Niswender (9), who used a radioiodinated tryptamine derivative. Geffard *et al.* (10) and Takahashi *et al.* (6) also used the same radioiodinated tryptamine derivative as tracer in their respective melatonin RIA. Direct iodination of melatonin for use as a radiolabeled tracer was first described by Vakkuri *et al.* (2). By using the iodo-gen with the chloramine-T method described by Greenwood *et al.* (11) for direct iodination of melatonin, they established by mass spectrometry and nuclear magnetic resonance methods that iodinated melatonin is 2-iodomelatonin. Using 2-[^{125}I]iodomela-

tonin as radiolabeled ligand, melatonin receptors have been identified in human, rat, and hamster brains and in chicken retina. With 2-[^{125}I]iodomelatonin as radiolabeled ligand and a polyclonal rabbit antimelatonin antiserum, a 2-day equilibrium double-antibody assay procedure was developed for quantification of melatonin in chicken pineal, serum, and medium samples. By using 2-[^{125}I]iodomelatonin, costly and cumbersome scintillation counting procedures can be avoided. However, this modified direct assay retains the specificity and sensitivity described in previously reported assays. The cross-reactivity data confirmed that the antiserum is specific for measuring melatonin. The recovery data, and the parallel displacement between the melatonin standard and chicken pineal, serum, and incubation medium samples, provide validation for this modified RIA method.

Using this direct melatonin RIA, rhythmic release of melatonin into the incubation culture medium can be demonstrated with chicken pineal glands maintained in either the static or superfusion system. This rhymic release of melatonin is maintained for three light:dark cycles in the static system and for at least seven light:dark cycles in the superfusion system. In the static system, there is a consistent lower amplitude of the first nighttime peak of melatonin in all experiments. Since samples for the first night were not collected in the static system experiments, the first light:dark cycle of melatonin is actually the second light:dark cycle for the pineal glands. These data suggest that pineal melatonin rhythms resume quicker in superfusion than in static culture systems. In previous studies using NAT activity as an assay for pineal rhythmicity *in vitro*, Binkley *et al.* (3) and Deguchi (4) both reported strong pineal NAT circadian rhythms during the first light:dark cycle but they were unable to find persistent circadian rhythms in later light:dark cycles. The negative NAT activity reported by Binkley *et al.* (3) may be due in part to the difficulty in measuring a low amplitude rhythm. Kasal *et al.* (5) reported that they observed two cycles of NAT rhythms in constant darkness. There is no apparent explanation why it has taken two light:dark cycles in the static system and one light:dark cycle in the superfusion system before pineal melatonin rhythms resume in this study. In all cases, the robust low daytime and high nighttime levels of melatonin rhythm resumed on the second in the superfusion system or third light:dark cycle in the static system and persisted to the end in all experiments.

In the superfusion culture system for chicken pineal glands, strong and robust circadian rhythms of melatonin release into the perfusate were observed. This strong daytime low and nighttime high melatonin rhythm persists for at least seven light:dark cycles. The present data agree with those reported by Takahashi and his colleagues (6, 12), who observed a strong circadian rhythm of melatonin release for as long as five light:dark cycles from chicken pineal glands maintained in a superfused *in vitro* system. In almost every melatonin cycle in the superfused culture systems, the decrease in melatonin concentration preceded the lights-on transition and the increase of melatonin concentration began before the lights-off transition. The data in the static experiments were not as clear as those from the superfusion. In the static system, the melatonin cycle showed an increase in melatonin concentration preceding the lights-off transition only in the third light:dark cycle. The increase of melatonin concentration was delayed in the first and second light:dark cycles, suggesting that it has taken at least two light:dark cycles for the pineal glands to acclimate in the static culture system. These data also suggest that superfusion may be a better system to study pineal melatonin function.

Various biochemical and behavioral experiments have long since shown that the avian pineal gland contains a circadian oscillator that plays a major role in its overall temporal organization [13–15). It is clear from the present data and those reported by others that the chicken pineal gland melatonin cycles in a circadian fashion. The synthesis of melatonin in chicken is regulated by NAT, which also oscillates with a circadian rhythm *in vivo* (7, 14, 16, 17). In contrast, however, the rhythm of pineal NAT in rats is controlled by norepinephrine from the sympathetic fibers that innervate the pineal. Therefore, no NAT or melatonin circadian rhythm was observed when rat pineal glands were cultured *in vitro* (18, 19). Although the mechanism of the oscillation of pineal NAT and melatonin in the chicken pineal has been established, it is clear that the control mechanism is in part internal rather than external to the pineal. Also, it is clear that rhythmic light input maintains the amplitude and synchronizes the melatonin rhythm and that acute light exposure rapidly inhibits melatonin release and synthesis (6). Therefore, it would be interesting to investigate and compare the photoreceptor mechanisms mediating the NAT and melatonin in chicken pineal with those located in the retina. In addition, it is interesting to notice that the regulation of the rhythm of pineal function is under the control of sympathetic fiber innervation in higher vertebrates such as humans, bovines, and rodents, but the circadian oscillator in birds and fish seem to be located within the pineal glands. Therefore, the oscillation of pineal function also has an evolutionary implication.

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1. Skene DJ. Assay methodology: Advances and applications to pineal research. In: Trentini GP, De Gaetani C, Pevet P, Eds. Serono Symposia Publications from Raven Press. New York: Raven Press, Vol **44**: pp301–319, 1987.
2. Vakkuri O, Leppaluoto J, Vuolteenaho O. Development and validation of a melatonin radioimmunoassay using radioiodinated melatonin as tracer. Acta Endocrinologica **106**:152–157, 1984.
3. Binkley SA, Riebman JB, Reilly KB. The pineal gland: A biological clock in vitro. Science **202**:1198–1201, 1978.
4. Deguchi T. Circadian rhythm of serotonin *N*-acetyltransferase activity in organ culture of chicken pineal gland. Science **203**:1245–1247, 1979.
5. Kasal CA, Menaker M, Perez-Polo JR. Circadian clock in culture: *N*-Acetyltransferase activity in chick pineal glands oscillates in vitro. Science **203**:656–658, 1979.
6. Takahashi JS, Hamm H, Menaker M. Circadian rhythms of melatonin release from individual superfused chicken pineal glands *in vitro*. Proc Natl Acad Sci USA 77:2319–2322, 1980.
7. Wainwright SD, Wainwright LK. Regulation of cycle in chick pineal serotonin *N*-acetyltransferase activity in vitro by light. J Neurochem **35**:451–457, 1980.
8. Leung FC, Taylor JE, Steelman SL, Bennett CD, Rodkey JA, Long RA, Serio R, Weppelman RM, Olson G. Purification and properties of chicken growth hormone and the development of a homologous radioimmunoassay. Gen Comp Endocrinology **56**:389–400, 1984.
9. Rollag MD, Niswender GD. Radioimmunoassay of serum concentrations of melatonin in sheep exposed to different lighting regimens. Endocrinology **98**:182–189, 1976.
10. Geffard MR, Puizillout JJ, Delaage MA. A single radioimmunological assay for serotonin, *N*-acetyltransferase, 5-methoxytryptamine and melatonin. J Neurochem **39**:1271–1277, 1982.
11. Greenwood FC, Hunter WM, Glover JS. The preparation of ^{131}I-labelled human growth hormone of high specific radioactivity. Biochem J **89**:114–123, 1963.
12. Takahashi JS, Menaker M. Multiple redundant circadian oscillators within the isolated avian pineal gland. J Comp Physiol [A] **154**:435–440, 1984.
13. Axelrod I. The pineal gland: A neuroendocrine transducer. Science **184**:1341–1348, 1974.
14. Binkley S. Comparative biochemistry of the pineal gland of birds and mammals. Am Zool **16**:57–65, 1976.
15. Tamarkin L, Baird CJ, Almeida OFX. Melatonin: A coordinating signal for mammalian reproduction? Science **227**:714–720, 1985.
16. Binkley S, Geller EB. Pineal *N*-acetyltransferase in chickens: Rhythm persists in constant darkness. J Comp Physiol **99**:67–70, 1975.
17. Goto M, Oshima I, Tomita T, Ebihara S. Melatonin content of the pineal gland in different mouse strains. J Pineal Res **7**:195–204, 1989.
18. Moore RY, Klein DC. Visual pathways and the central neural control of circadian rhythm in pineal serotonin-*N*-acetyltransferase. Brain Res **71**:17–33, 1974.
19. Bowers CW, Dahm LM, Zigmond RE. The number and distribution of sympathetic neurons that innervate the rat pineal gland. Neuroscience **13**:87–96, 1984.