Proc. Natl. Acad. Sci. USA
Vol. 77, No. 4, pp. 2319–2322, April 1980
Neurobiology

Circadian rhythms of melatonin release from individual superfused chicken pineal glands *in vitro*

(oscillator/pacemaker/birds/organ culture)

JOSEPH S. TAKAHASHI*, HEIDI HAMM†, AND MICHAEL MENAKER*

Department of Zoology, University of Texas, Austin, Texas 78712

Communicated by Colin S. Pittendrigh, January 18, 1980

ABSTRACT **The pineal gland of birds contains one or more circadian oscillators that play a major role in overall temporal organization. We have developed a flow-through culture system for the isolated pineal by which we can measure the release of melatonin continuously from superfused glands over long periods of time. Chicken pineals release melatonin rhythmically, and these rhythms persist *in vitro* with a circadian oscillation. In light cycles the release of melatonin is strongly rhythmic; however, in constant conditions the amplitude of the rhythm is lower and appears to be damping. Light has at least two effects upon the isolated pineal: cyclic light input synchronizes the rhythm, and acute light exposure at night rapidly inhibits melatonin release. The cultured avian pineal clearly offers great potential as a model system for the study of vertebrate circadian oscillators and may open the way for an analysis of mechanism.**

Behavioral and biochemical experiments strongly suggest that the avian pineal gland contains, or is, a circadian oscillator that plays a major role in the overall temporal organization of the animal (for review, see ref. 1). Removal of the pineal eliminates the free-running circadian rhythms of locomotor activity and body temperature in the house sparrow (2, 3). Transplantation of pineal tissue into the anterior chamber of the eye rapidly restores rhythmicity in arrhythmic pinealectomized sparrows and transfers the phase of the donor's rhythm to the host (4–6). These results argue that the pineal gland functions as a pacemaker within the circadian system of the house sparrow.

Apart from the pineal's role in controlling behavioral rhythms, the biochemistry of the avian pineal itself is dramatically rhythmic. The melatonin content of chicken pineals oscillates in a circadian fashion (7, 8) and contributes to a rhythm of melatonin in the serum (9). The synthesis of melatonin in the chicken pineal appears to be regulated by the activity of the enzyme serotonin *N*-acetyltransferase (7), which oscillates with a circadian period *in vivo* (10). The regulation of the rhythm of pineal *N*-acetyltransferase—which in rats is under the control of norepinephrine from the sympathetic fibers that innervate the pineal (11–13)—appears to be different in birds (14). Although it is not clear how the oscillation in *N*-acetyltransferase activity in the avian pineal is generated, it is consistent with the data to conclude that the controlling mechanism is within rather than external to the pineal.

Recent experiments have established several important properties that are expressed by the isolated pineal gland in organ culture. Four laboratories have documented an increase and decrease in *N*-acetyltransferase activity in isolated pineals for at least 24 hr in organ culture (15–19).

Kasal *et al.* (17) have shown that the rhythm persists for two cycles in constant darkness with a circadian period *in vitro*. However, the difficulty in measuring rhythms in populations of isolated glands (unavoidable when *N*-acetyltransferase activity is used as an end point) and the fact that the rhythm appears damped have precluded a definitive conclusion that the avian pineal is a self-sustained oscillator. We have developed a flow-through pineal organ culture method which allows us to measure the release of melatonin continuously from individually isolated pineal glands. We have found a robust rhythm of melatonin release in individual chicken pineal glands that persists *in vitro* for at least 5 days. This technique allows us to study, *in vitro*, circadian rhythms in the avian pineal in detail and may prove to be a useful model system for studying the cellular and subcellular mechanisms that underlie rhythmicity in a vertebrate circadian oscillator.

MATERIALS AND METHODS

Chemicals. Carrier-free, high-specific-activity ^{125}I was purchased from Amersham. Melatonin was purchased from Sigma. Normal rabbit gamma globulin was purchased from Cappel Laboratories (Cochranville, PA). Rabbit anti-melatonin antibody was graciously supplied by Mark Rollag.

Animals. Newly hatched chicks (*Gallus domesticus*, White Leghorn, Babcock strain) were purchased from a local supplier and raised under alternating light (12 hr)/dark (12 hr) lighting conditions. Food (Purina Chick Startena) and water were continuously available.

Pineal Culture. At 5–8 weeks of age, chickens were sacrificed during the day, approximately 4 hr before lights-out. They were decapitated, and the heads were rinsed in a disinfectant solution. The cap of the skull was cut away and the pineal was dissected free from the dura. Several cuts were made in each gland to facilitate diffusion. Pineals were rinsed three times in medium 199 containing 1000 units of penicillin and 1000 μg of streptomycin per ml. Individual pineals were then placed immediately into the sterile flow-through culture apparatus (Fig. 1). Each pineal was cultured in a borosilicate glass chamber; a stainless steel mesh basket held the gland. Culture medium was infused into the basket, and surface tension ensured that a drop of medium always bathed the gland. Typically, drops of medium slowly formed at the bottom of the basket and flowed into the output tube. Sterile 95% oxygen/5% carbon dioxide was continuously fed into the chambers. The gas flow facilitated the drainage of the chamber (flushing time, ≈30 sec). Timed samples of the perfusate were collected automatically with a fraction collector. Collecting tubes contained 75 μg of $NaHCO_3$ at pH = 10 to retard contamination of the culture medium; this was effective for more than 48 hr but samples were collected and frozen every 10–14 hr.

Three formulations of culture media were used in these ex-

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Interdisciplinary Program in Neurosciences, Department of Biology, University of Oregon, Eugene, OR 97403.
† Present address: Laboratory of Molecular Biology, University of Wisconsin, Madison, WI 53703.

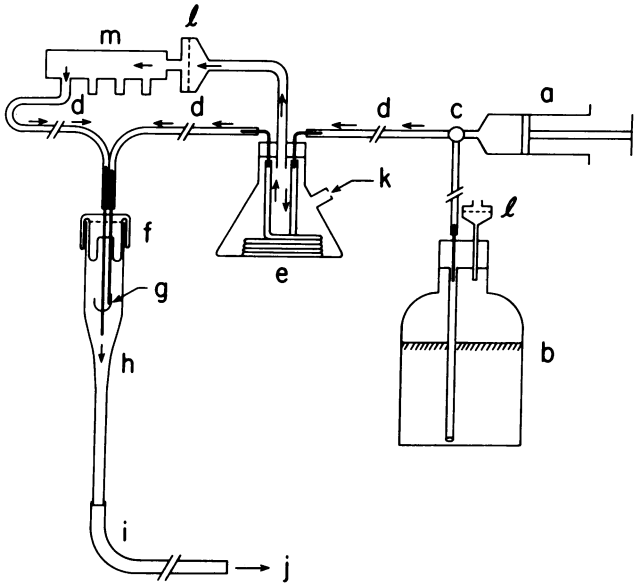

FIG. 1. Diagram of flow-through organ culture apparatus. a, Syringe pump for infusing culture medium; b, reservoir bottle for culture medium; c, three-way stopcock; d, Tygon microbore tubing (0.020 in. inside diameter × 0.060 in. outside diameter; 0.5 × 1.5 mm); e, chamber for gassing culture medium, using Silastic tubing; f, rubber serum stopper; g, stainless steel mesh basket for holding pineal tissue (a short 25-gauge stainless steel tube was used for delivering medium to the basket and a longer tube was used to support the basket and to deliver gas to the chamber); h, borosilicate glass chamber made from a pasteur pipette; i, Tygon microbore tubing (0.050 in. inside diameter × 0.090 in. outside diameter; 1.3 × 2.3 mm); j, output collected in a fraction collector; k, input for humidified 95% O_2/5% CO_2; ℓ, Millipore filter (0.22 μm); m, manifold for distributing filtered gas to culture chambers. The arrows indicate flow of culture medium (a, c, d, e, d, g, h, i, j) and 95% O_2/5% CO_2 gas (k, e, ℓ, m, d, h, i, j).

periments; all were obtained from GIBCO: (*i*) medium 199 with 25 mM Hepes buffer, Hanks' salts, and L-glutamine; (*ii*) medium 199 with Earle's salts, L-glutamine, and 100 units of penicillin and 100 μg of streptomycin per ml; (*iii*) the same medium as in (*ii*) with the addition of 10% fetal bovine serum. Culture medium was infused by a Harvard Apparatus syringe pump fitted with 10-ml syringes. A reservoir bottle containing enough medium for the entire experiment was attached by a three-way valve to refill the syringes and was kept in an ice bath. We used perfusion rates of 0.25, 0.5, and 1.0 ml/hr. The residence time for melatonin (the amount of time required to flush out one-half the concentration of melatonin) with the 1.0 ml/hr rate was ≈10 min. The medium was gassed before it entered the culture chamber by passing it through small-diameter (large surface area) Silastic (Dow Corning) tubing gassed with 95% oxygen/5% carbon dioxide. Once a pineal gland was placed in the culture chamber, the entire apparatus was sealed and not opened for the duration of the experiment. The entire culture system was housed in a large environmental chamber at 37°C ± 0.2°C. This fluctuation was due to hysteresis in the thermostat and does not reflect day/night temperature changes.

Radioimmunoassay. Melatonin levels in the pineal perfusates were determined by radioimmunoassay using the method of Rollag and Niswender (20). The following modifications to the original assay were used. An equilibrium assay procedure was utilized for quantification of melatonin. Appropriate volumes (1–2 μl) of pineal perfusate were diluted with 0.01 M phosphate buffer (pH 7.0) containing 0.1% gelatin (Eastman) and 0.9% NaCl to a total volume of 500 μl. In an ice bath, 100 μl of the assay buffer containing the ^{125}I-labeled melatonin analogue (32,000 cpm) and 200 μl of a 1:64,000 dilution of antiserum (rabbit 1055) in 0.01 M phosphate buffer, pH 7.0/0.9% NaCl/0.05 M EDTA/0.005% normal rabbit gamma globulin were added to each assay tube. The reaction mixture was incubated for 48 hr at 4°C. Then, 3.0 ml of ice-cold 95% ethanol was added to each tube in an ice bath. After 2–10 min, the reaction mixture was centrifuged at 2000 × *g* for 30 min at 4°C. The supernatant was decanted, and radioactivity in the precipitate was measured in a Nuclear-Chicago gamma spectrometer. The potency estimates for melatonin were calculated with computer programs developed by Rodbard and associates (21).

Radioimmunoassay Validation. Preliminary experiments showed that there were no interfering substances in any of the three culture medium formulations. Because we were primarily interested in relative differences in pineal melatonin (or methoxyindole) concentrations rather than in absolute levels, we validated the assay without extraction of the samples. This results in a significant saving in both time and labor and, more importantly, allows us to assay large numbers of samples routinely which is necessary for high resolution in the time domain. Melatonin (0, 10, 25, 50, 100, 250, and 500 pg/tube; four replicates at each level) added to pooled medium 199 pineal perfusate collected at night was quantitatively recovered (linear regression: $y = 1.10x + 185$; $r = 0.99$; y-intercept, 185 pg, represents the endogenous concentration of melatonin in the pineal perfusate sample). Serial dilutions of pooled day and pooled night medium 199 pineal perfusate samples resulted in parallel inhibition curves for "percentage bound" values between 10 and 90%. The lower and upper limits of the assay were ≈2.5 and 500 pg/tube, respectively. Fifty percent inhibition was typically produced upon addition of 35 pg of melatonin standard per tube. Pooled medium 199 pineal perfusate samples (six replicates each) with mean melatonin concentrations of 16.7, 76.6, and 415.6 ng/ml had intra-assay coefficients of variation of 13.8, 9.9, and 11.8%, respectively. The respective interassay coefficients of variation were 8.5, 6.4, and 7.1% (six assays).

RESULTS

Chicken pineal glands maintained in the flow-through culture system released melatonin into the perfusate rhythmically (Fig. 2). In this experiment, the light/dark cycle was continued *in vitro* with the same phase as the previous *in vivo* light cycle. Melatonin concentration in the superfusate reached peak levels during the dark and minimal levels during the light (3- to 15-fold increase at night). Individual chicken pineals released large quantities of immunoreactive melatonin. In this system, pineals from chickens between 5 and 8 weeks old produced 75–100 ng of radioimmunoassayable melatonin per hour at night and 10–30 ng per hour during the day. The total amount of melatonin produced averaged 1000 ng/24 hr. Although there are quantitative differences in the amplitude of the rhythms among glands, the general pattern of the rhythm is remarkably stable and uniform. In almost every case the decrease in melatonin concentration preceded the lights-on transition, and the increase began before lights-off. Thus, the rhythm does not appear to be entirely driven by the light cycle but rather shows anticipatory behavior which may reflect the entrainment of an oscillator. There was very little damping of the rhythm until the fourth or fifth peak. The phase of the rhythm is similar to that found *in vivo* in the pineal (7).

To investigate the endogenous nature of the pineal melatonin rhythm, pineal melatonin production was examined under constant dim red light from a photographic safe light (1–5 lux).

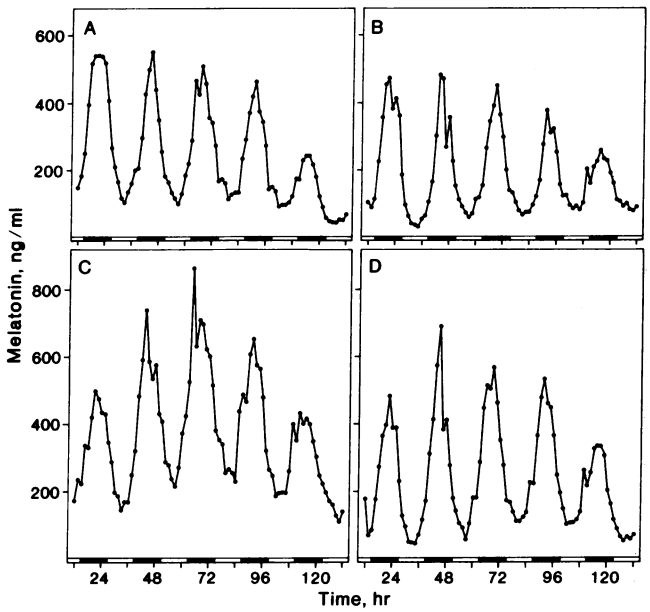

FIG. 2. Rhythms of melatonin release from four different individually isolated chicken pineal glands cultured in a flow-through superfusion system. The *in vitro* 12-hr light (350–500 lux cool white fluorescent) and dark (0 lux) cycle is indicated at the bottom of each panel. Samples of perfusate were collected for 90 min. Concentration of melatonin in the culture medium (formulation *i*; see text) was determined by radioimmunoassay. Each point represents a single determination and is plotted at the start of the collection interval. The flow rate in this experiment was 0.25 ml/hr, and the culture medium was not gassed before it entered the chamber.

Fig. 3 shows the results of two different experiments, both performed under identical culture conditions using pineals from the same group of chickens. In a 60-hr experiment with the 12-hr light/dark cycle, there was a high-amplitude melatonin rhythm on both days. In contrast, in the absence of a light/dark cycle, there was one high-amplitude peak in melatonin concentration followed by three lower-amplitude peaks at about 24-hr intervals. In constant conditions there was an oscillation in pineal melatonin which persisted for at least four cycles; however, it was heavily damped compared to the rhythm present under light/dark conditions. The damping of the rhythm is probably not due to unfavorable culture conditions because the rhythm did not damp when a light cycle was present. We have repeated these experiments with each of the three different formulations of medium 199 and have not found any significant differences. Although we cannot exclude the possibility of inadequate culture conditions, these experiments suggest that the isolated chicken pineal requires cyclic light input to be strongly rhythmic.

We have also investigated the effects of light upon melatonin release at night. In three experiments we exposed chicken pineals to 1 or 2 hr of light at the peak of the melatonin rhythm on the first night (data now shown). Light rapidly inhibited the release of melatonin from the cultured pineals. A 50% decrease of the melatonin concentration in the perfusate occurred in 20–30 min (1.0 ml/hr flow rate). The rapid inhibition of melatonin by light in our culture system is similar to the time course of inhibition by light of *N*-acetyltransferase in static organ culture reported by Deguchi (18). The general correlation between the activity of *N*-acetyltransferase (17, 18) and the release of melatonin suggests that melatonin concentration in our culture system closely reflects the activity of *N*-acetyltransferase.

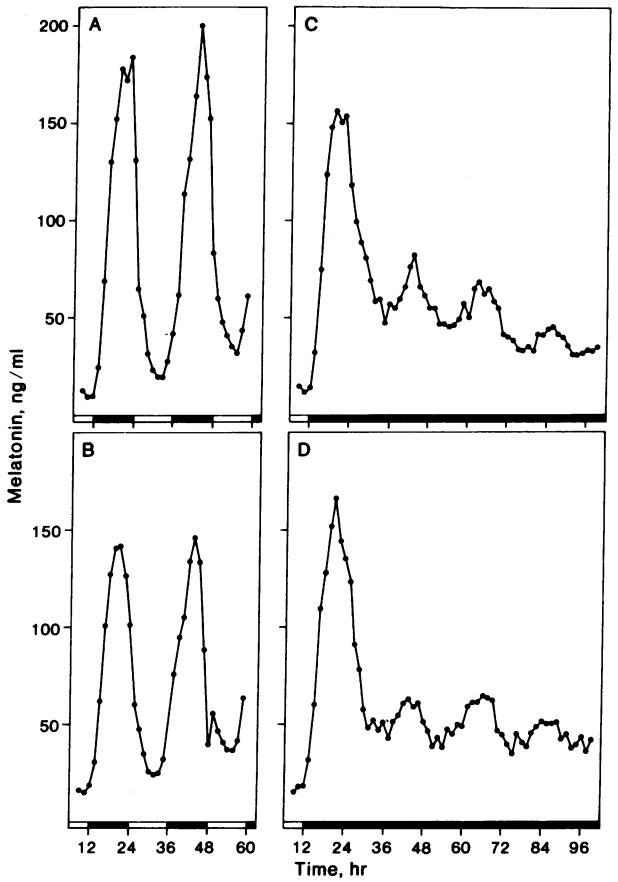

FIG. 3. Rhythms of melatonin release in a light/dark cycle (*A* and *B*) and in constant conditions (*C* and *D*). The light treatments are indicated at the bottom of each panel. (*A* and *B*) Two individual pineals from a 60-hr experiment with a 12-hr light (350–500 lux cool white fluorescent) and 12-hr dark (1–5 lux red light) cycle. (*C* and *D*) Two individual pineals from a 96-hr experiment in constant dim red illumination (1–5 lux). Pineals in both experiments came from the same group of chickens. Culture medium was formulation *iii*. The flow rate was 0.5 ml/hr (the flow rate in these experiments was twice as high as that in the experiments shown in Fig. 2, which accounts for most of the difference in the melatonin concentration in the perfusate). Collection interval and points plotted are as in Fig. 2.

DISCUSSION

We have shown that robust rhythms of melatonin release can be measured continuously from individual pineal glands in a flow-through culture system. By using this method it is feasible to assay pineal rhythms of melatonin *in vitro* for long periods of time and with high resolution in the time domain. In constant conditions the rhythm persists in culture for at least four cycles with a circadian period; however, the rhythm appears to be heavily damped. It does not seem likely that damping is due to poor culture conditions because in light/dark cycles the rhythm is not damped and persists with high amplitude. Previous experiments using *N*-acetyltransferase activity as an assay for pineal rhythmicity *in vitro* have yielded equivocal results in constant conditions. Although Kasal *et al.* (17) were able to find two cycles of *N*-acetyltransferase rhythms in constant darkness, Binkley *et al.* (16) and Deguchi (18) reported that they were unable to find persistent circadian rhythms. Our results suggest that these negative findings are due in part to the difficulty in measuring low-amplitude rhythms in experiments assaying population rhythms. This illustrates the advantage of assaying rhythmicity in individual glands. However, our results show that the damping of the rhythm seen in the

static culture experiments is probably not a result of population asynchrony but rather of a process that occurs within an individual pineal gland. Deguchi (22) has reported that rhythms of *N*-acetyltransferase activity persist in dispersed pineal cell cultures exposed to a 12-hr light/dark cycle and perhaps in constant darkness. Although his results do not exclude the possibility of cellular interactions, they suggest that circadian rhythmicity in the pineal may be a cellular property. If circadian rhythmicity is a cellular property and many pineal cells are circadian oscillators, then it is possible that the damping of the rhythm within a gland, which we see in our experiments, may result from asynchrony of individual cellular oscillators.

Whatever mechanism underlies the damping of the chicken pineal melatonin rhythm in constant dim red light *in vitro*, it is clear that rhythmic light input maintains the amplitude and synchronizes the rhythm. In addition to the synchronizing effects of light, acute light exposure at night, when melatonin levels are high, rapidly inhibits melatonin release and synthesis (cf. ref. 18). Thus, light acts on the isolated pineal in two ways: as a synchronizing agent and as an inhibitor of melatonin production. The nature of the photoreceptor(s) mediating these responses has not been studied, and it will be of interest to compare pineal photoreceptor mechanisms with those of the retina.

We have recently reviewed present knowledge about the organization of circadian systems in birds (1) and thus need only discuss this topic briefly. The pineal plays a dominant role in the circadian system of the house sparrow, *Passer domesticus*. It is necessary for the persistence of circadian rhythmicity; pineal transplants restore circadian rhythmicity in arrhythmic pinealectomized hosts; and pineal transplants determine the phase of the restored rhythm (2–6). These experiments and others strongly suggest that the pineal of the sparrow has a pacemaking function and that it is hormonally coupled to the rest of the circadian system. In chickens, there is a circadian rhythm of melatonin in the pineal and the serum (7, 8) and this serum rhythm is undetectable after pinealectomy (9). The results reported here unequivocally show that a rhythmic hormonal signal (melatonin) is released from the chicken pineal. In sparrows, continuous administration of melatonin shortens the free-running period length of the activity rhythm at low doses and induces continuous activity at higher doses (23). Furthermore, daily injections of melatonin synchronize the locomotor rhythms of pinealectomized starlings (24). Thus, the rhythmic release of melatonin seen in our organ cultures is likely to be of real biological significance as an output pathway for coupling the pineal with the rest of the circadian system.

The reported effects of pinealectomy on circadian rhythmicity in birds are variable and range from arrhythmicity in house sparrows (2, 3) to no effect in chickens (25) and Japanese quail.[‡] The lack of an effect of pinealectomy in chickens is especially intriguing because pineals from chickens oscillate when isolated in organ culture. The behavioral work suggests that interspecific differences in the circadian organization of birds may exist (1) and raises the possibility that there are species differences in the circadian properties of the pineal itself. Its clearly established role as a pacemaker suggests that the pineal of the house sparrow may retain a more robust circadian oscillation *in vitro* under constant conditions than does that of the chicken.

[‡] Simpson, S. M. & Follett, B. K. (1980) *Proceedings of the 17th International Congress of Ornithology (Berlin)*, in press.

Although we now recognize that circadian systems provide one of the most important temporal frameworks for neural and hormonal organization, we know very little about the physiology or biochemistry of the oscillators that compose such systems or about the interactions among these oscillators (26–28). The avian pineal is the only vertebrate circadian oscillator that it has so far been possible to study *in vitro*. It is therefore the only available model system from which we can hope to learn about the internal mechanism of vertebrate circadian oscillators. As such, it should repay further study.

Special thanks are extended to Catherine Norris for performing assays. We thank M. Graham and Dr. C. Desjardins for supplying computer analysis programs, Dr. F. Bronson for laboratory facilities, and Dr. M. Rollag for the melatonin antibody. This research was supported by a National Science Foundation Graduate Fellowship to J.S.T. and National Institutes of Health Grants HD-03803 and HD-07727 to M.M.

1. Takahashi, J. S. & Menaker, M. (1979) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **38,** 2583–2588.
2. Gaston, S. & Menaker, M. (1968) *Science* **160,** 1125–1127.
3. Binkley, S. Kluth, E. & Menaker, M. (1971) *Science* **174,** 311–314.
4. Zimmerman, N. H. & Menaker, M. (1975) *Science* **190,** 477–479.
5. Menaker, M. & Zimmerman, N. H. (1976) *Am. Zool.* **16,** 45–55.
6. Zimmerman, N. H. & Menaker, M. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 999–1003.
7. Binkley, S., MacBride, S. E., Klein, D. C. & Ralph, C. L. (1973) *Science* **181,** 273–275.
8. Ralph, C. L., Pelham, R. W., MacBride, S. E. & Reilly, D. P. (1974) *J. Endocrinol.* **63,** 319–324.
9. Pelham, R. W. (1975) *Endocrinology* **96,** 543–546.
10. Binkley, S. & Geller, E. B. (1975) *J. Comp. Physiol.* **99,** 67–70.
11. Axelrod, J. (1974) *Science* **184,** 1341–1348.
12. Axelrod, J. & Zatz, M. (1977) in *Biochemical Actions of Hormones*, ed. Litwack, G. (Academic, New York), Vol. 4, pp. 249–268.
13. Klein, D. C. (1978) in *The Hypothalamus*, eds. Reichlin, S., Baldessarini, R. J. & Martin, J. B. (Raven, New York,) pp. 303–326.
14. Binkley, S. (1976) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **35,** 2347–2352.
15. Deguchi, T. (1978) in *Integrative Control Functions of Brain*, ed. Ito, M. (Kodansha Scientific, Tokyo, Japan), Vol. 1, pp. 345–347.
16. Binkley, S. A., Reibman, J. B. & Reilly, K. B. (1978) *Science* **202,** 1198–1201.
17. Kasal, C. A., Menaker, M. & Perez-Polo, J. R. (1979) *Science* **203,** 656–658.
18. Deguchi, T. (1979) *Science* **203,** 1245–1247.
19. Wainwright, S. D. & Wainwright, L. K. (1979) *Can. J. Biochem.* **57,** 700–709.
20. Rollag, M. D. & Niswender, G. D. (1976) *Endocrinology* **98,** 482–489.
21. Rodbard, D. & Hutt, D. M. (1974) in *Symposium on RIA and Related Procedures in Medicine, International Atomic Energy Agency, Vienna* (Unipub, New York), Vol. 1, pp. 165–189.
22. Deguchi, T. (1979) *Nature (London)* **282,** 94–96.
23. Turek, F. W., McMillan, J. P. & Menaker, M. (1976) *Science* **194,** 1441–1443.
24. Gwinner, E. & Benzinger, I. (1978) *J. Comp. Physiol.* **127,** 209–213.
25. MacBride, S. E. (1973) Dissertation (Univ. of Pittsburgh, Pittsburgh, PA).
26. Menaker, M., Takahashi, J. S. & Eskin, A. (1978) *Annu. Rev. Physiol.* **40,** 501–526.
27. Block, G. D. & Page, T. L. (1978) *Annu. Rev. Neurosci.* **1,** 19–34.
28. Rusak, B. & Zucker, I. (1979) *Physiol. Rev.* **59,** 449–526.