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ALTERATIONS OF PINEAL GLAND BIORHYTHMS BY N-METHYL-3-PIPERIDYL BENZILATE (JB336)[1]

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ABSTRACT

MERRITT, JAMES H. AND THOMAS S. SULKOWSKI: Alterations of pineal gland biorhythms by N-methyl-3-piperidyl benzilate (JB336). J. Pharmacol. Exp. Ther. **166**: 119–124, 1969. N-methyl-3-piperidyl benzilate (JB336), an anticholinergic psychotogenic agent, disrupts the circadian rhythm of serotonin in the rat pineal gland when the drug is administered at 0800 hours. The dose used, 1 mg/kg, has no effect on whole rat brain serotonin. JB336 alters hydroxyindole-O-methyltransferase activity at various times of the 24-hr day. Protein and total ribonucleic acid content of the rat pineal gland are found to have circadian rhythms. These rhythms are also disrupted by a single injection of 1 mg of JB336 per kg administered at 0800 hours.

The biorhythms of serotonin and norepinephrine content are controlled by sympathetic innervation to the pineal gland (Fiske, 1964; Wurtman *et al.*, 1967). These rhythms can be abolished by interrupting sympathetic transmission to the pineal (Snyder *et al.*, 1964; Fiske, 1964; Wurtman *et al.*, 1967). Exogenous control of norepinephrine variation appears to be initiated by light perceived by the retina (Wurtman *et al.*, 1967), whereas the serotonin rhythm appears to be endogenously controlled but triggered by light (Snyder *et al.*, 1965c). Some of the enzymes associated with metabolism and synthesis of the biogenic amines exhibit a periodicity of their activities in pineal tissue (Axelrod *et al.*, 1965; McGeer and McGeer, 1966). Activities of 5-hydroxytryptophan decarboxylase (Snyder and Axelrod, 1964) and hydroxyindole-O-methyltransferase (Axelrod *et al.*, 1965) are altered by continuous light or darkness. As with pineal amine content, interruption of sympathetic innervation abolishes the effect of light on these enzymic activities (Axelrod *et al.*, 1966; Snyder *et al.*, 1965a).

N-methyl-3-piperidyl benzilate (JB336) is an anticholinergic drug with marked psychotogenic activity (Abood *et al.*, 1958). Psychotogenic piperidyl benzilates induce electroencephalogram (EEG) synchrony and block physostigmine activation of the EEG (White and Carlton, 1963). It is concluded that cholinergic mechanisms involved in the EEG-arousal phenomenon are blocked by these piperidyl benzilates (Brimblecombe and Green, 1968).

Information relative to environmental lighting is transmitted to the pineal gland from the central nervous system *via* the superior cervical ganglia and could be routed over cholinergic synapses. This study describes alterations of biorhythms by an anticholinergic drug.

METHODS. Male Sprague-Dawley rats[2] (300–400 g) were used throughout the study. The animals were maintained, five to a cage, in a light-tight room at a constant humidity and temperature. An automatic timer provided 12 hr of light and 12 hr of darkness. Light was provided by fluorescent lamps which yielded about 35 foot-candles at the cage levels. All animals were allowed to adapt to this environment for at least 2 weeks before they were used.

The animals were injected i.p. with 1 mg of JB336[3] per kg at 0800 hours. Control animals

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[2] The animals involved in this study were maintained in accordance with the Guide for Laboratory Animal Facilities and Care as published by the National Academy of Sciences—National Research Council.

[3] JB336 was obtained from the Food Machinery Corporation, Chemicals Division, Princeton, N.J.

were injected with saline. They were sacrificed after treatment by cervical dislocation at 1200, 1800, 2400 and 0600 hours (4, 10, 16 and 22 hr, respectively, after injection) and the pineal glands were removed. After removal, the glands were immediately weighed on a Cahn Electrobalance and homogenized in a glass conical centrifuge tube with a tapered nylon pestle. Serotonin was assayed by the method of Snyder *et al.* (1965b). Hydroxyindole-O-methyltransferase (HIOMT) activity was assessed utilizing the method of Axelrod *et al.* (1965). RNA was measured as described by Shibko *et al.* (1967), utilizing ultraviolet absorption. Protein was determined with an automated adaptation of the Lowry method (Lowry *et al.*, 1951). Ribonucleic acid (RNA) and protein were measured in the same pineal, whereas each of the other chemical parameters was measured in separate pineals.

RESULTS. After administration of 1 mg of JB336 per kg, the rats were moderately hyperactive and exhibited moderate mydriasis. Food and water intake was not significantly different from control animals.

Modification of pineal gland serotonin diurnal variation. Rats maintained in an environment of alternating 12-hr periods of light (0700–1900 hours) and darkness (1900–0700 hours) exhibited a diurnal variation of the amplitude described by Quay (1963). The peak is reached at noon with a continuous fall during the dark period to a nadir at 2400 hours (fig. 1). To examine the effect of a psychotogenic piperidyl benzilate on the circadian periodicity of pineal serotonin, the rats were injected with 1 mg of JB336 per kg at 0800 hours. The animals were killed at intervals thereafter and their pineals were assayed for serotonin content. Treatment with JB336 resulted in a decrease during the 4 hr after injection (fig. 1) so that the 1200-hours value was significantly lower than control ($P < .01$). The level then rose sharply to a peak at 1800 hours nearly as high as that exhibited by the control animals at 1200 hours. The pineal serotonin value of the treated animals at 1800 hours was significantly different from the control value ($P < .01$). Thereafter the serotonin content fell to its lowest point at midnight and rose again at 0600 hours in a fashion similar to controls.

JB336 at doses of 1 mg/kg and 10 mg/kg had no effect on whole rat brain serotonin when serotonin was assayed 2 hr after administration of the drug. These data seem to indicate that JB336 does not effect a general release of serotonin in the central nervous system.

Alteration of hydroxyindole-O-methyltransferase (HIOMT) activity. Axelrod *et al.* (1965) found a diurnal variation in the activity of pineal HIOMT. On the other hand, Quay (1967) reported that he was unable to demonstrate a circadian rhythm in pineal HIOMT. In our study HIOMT activity exhibited little variation over a 24-hr period under conditions of diurnal lighting (fig. 2). To investigate the possibility of alteration of HIOMT by JB336, the rats were treated with 1 mg/kg at 0800

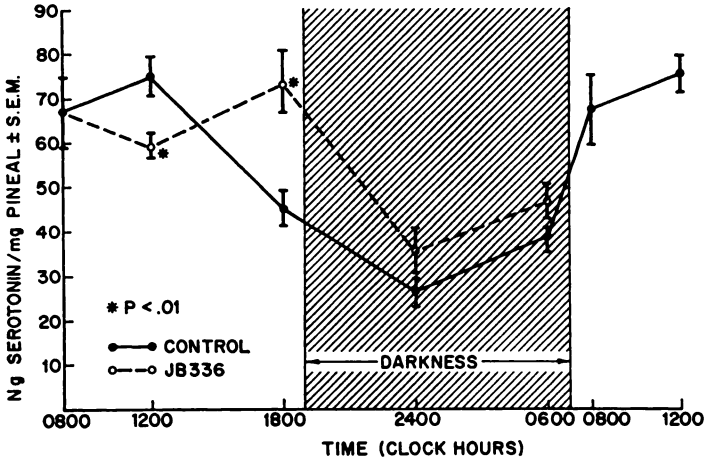

FIG. 1. Effect of JB336 on pineal serotonin rhythm. Groups of nine to 10 animals were injected with 1 mg of JB336 per kg or saline and sacrificed at the various times indicated. The P value represents significance of the difference between control and treated means for the given hour.

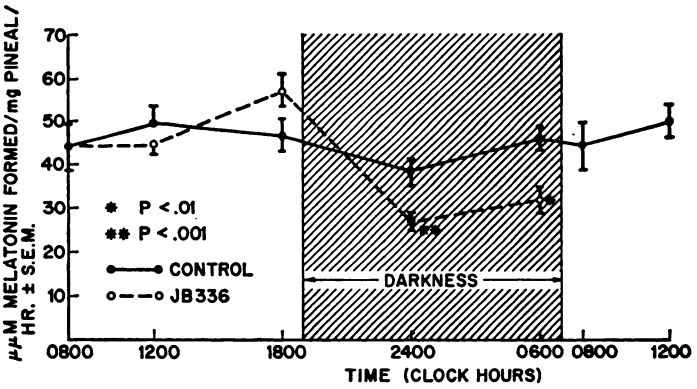

FIG. 2. Effect of JB336 on pineal gland hydroxyindole-O-methyltransferase activity. Groups of seven to 10 animals were injected with 1 mg of JB336 per kg or saline and sacrificed at the various times indicated. The P values represent significance of the difference between control and treated means for the given hour.

hours, and enzymic activity was measured at intervals afterwards. HIOMT activity was increased slightly at 1800 hours (10 hr postinjection) above control. Enzyme activity then declined sharply during the next 6 hr, so that at 2400 hours the activity was significantly reduced when compared with control activity ($P < .001$). At 0600 hours (22 hr after treatment), the activity was significantly less than the control activity ($P < .01$). From these data, it seems possible that pineal control of HIOMT is altered by JB336.

Diurnal variation of pineal protein and RNA content and effect of JB336. A diurnal variation in mitotic activity and in parenchymal mean nuclear and nucleolar dimensions is seen in rat pineal (Quay and Renzoni, 1966), and a daily variation in pineal gland weight has also been observed (Axelrod *et al.*, 1965). We were unable to show a change in weights of pineal glands among animals sacrificed at 0600, 0800, 1200, 1800 and 2400 hours, nor did injection of 1 mg of JB336 per kg alter pineal weight.

In order to determine whether there is a rhythmicity in pineal protein and RNA content when rats are maintained in diurnal lighting, groups of animals were sacrificed at five different times during a 24-hr period, and RNA and protein were determined. Pineal protein exhibited a definite variation during the day. A peak occurred at 1800 hours, immediately preceded by a trough at 1200 hours (fig. 3). It is suggested that maintenance of peak noontime levels of serotonin is somehow dependent upon RNA formation (Snyder *et al.*, 1967). In rat brain, serotonin content and RNA polymerase activity vary inversely (Siegel and Salinas, 1968). RNA levels responded with a rhythmicity when pineal glands were assayed for total RNA content at various times throughout the day. A nadir was observed at 1200 hours, with a peak occurring at 0800 hours (fig. 4). This RNA cycle is approximately 180° out of phase with the pineal protein cycle. The ratio, protein/RNA, is constant in metabolically active tissue such as liver in various nutritional states (Banks *et al.*, 1964). When protein/RNA ratios are plotted against time of day, a circadian rhythm is evident (fig. 5). The peak of this diurnal variation occurs at 1800 hours and the trough at 0800 hours. These data suggest that overall activity of the pineal gland varies widely during the 24-hr day and involves more than changes in biogenic amine levels.

In order to determine the effect of JB336 on the diurnal variation of protein and RNA, rats were injected with the drug at 0800 hours and sacrificed 4, 10, 16 and 22 hr later. After a single injection of JB336, pineal protein levels declined and remained depressed until 16 hr posttreatment (2400 hours). At this time the protein content returned to control value (fig. 3). When pineal RNA content was measured after JB336 treatment, RNA levels remained essentially within control limits until 22 hr (0600 hours) after injection, at which time the total RNA was depressed (fig. 4). Protein/RNA

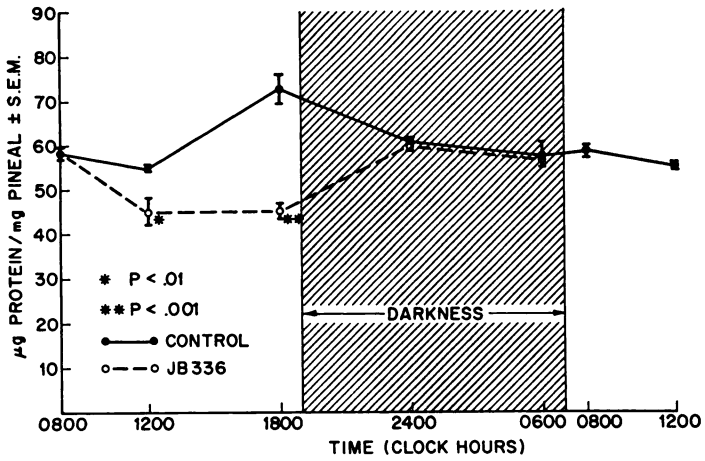

FIG. 3. Effect of JB336 on pineal gland protein. Groups of six to 10 animals were injected with 1 mg of JB336 per kg or saline and sacrificed at the various times indicated. The P values represent significance of the difference between control and treated means for the given hour. The 1800-hours control value is significantly different ($P < .05$) from all other control values.

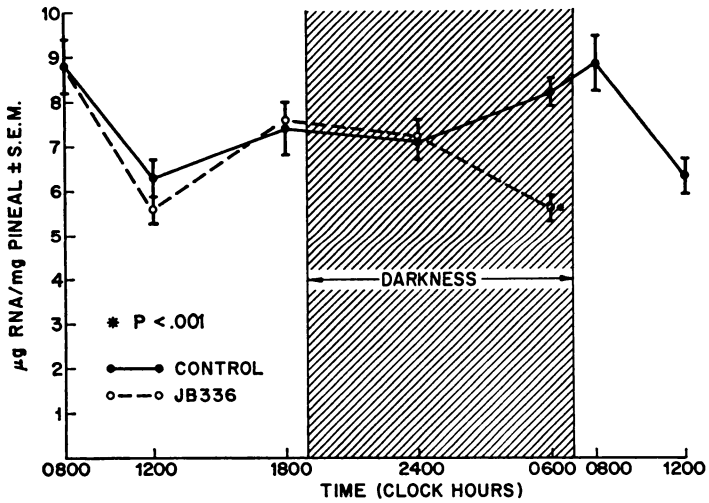

FIG. 4. Effect of JB336 on pineal gland RNA. Groups of six to 10 animals (except for control at 0600, where $N = 19$) were injected with 1 mg of JB336 per kg and sacrificed at the times indicated. The P value represents significance of the difference between control and treated means for the given hour. The 0600-hours value is significantly different ($P < .05$) from the 1200-hours value, and the 0800-hours value is significantly different ($P < .05$) from the 1200-hours and 2400-hours values.

ratios after JB336 are seen to be almost the reciprocal of the control ratios. After JB336 treatment, the lowest ratio occurs at 1800 hours (fig. 5). These results indicate that pineal control of protein and RNA metabolism and, possibly, overall pineal gland activity are altered by a psychotogenic piperidyl benzilate.

DISCUSSION. The foregoing data indicate that the psychotogenic piperidyl benzilate JB336 disrupts biorhythms within the pineal gland. The well known rhythmicity of serotonin in the pineal gland (Quay, 1963; Snyder and Axelrod, 1964) is shifted so that the peak occurs 6 hr later than control. Snyder *et al.* (1967) have suggested that the rapid decline in pineal serotonin after the onset of darkness may be caused by a release of serotonin from the bound form. It seems reasonable to assume that this release would be under control from the sympathetic nervous system since abolition of sympathetic

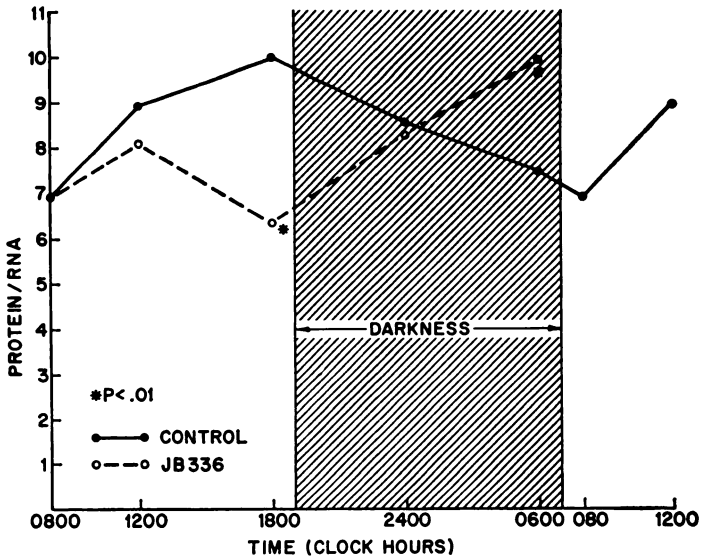

FIG. 5. Effect of JB336 on protein/RNA ratio in rat pineal gland. The P value represents significance of the difference between control and treated means for the given hour.

innervation of the pineal also abolishes the diurnal pineal serotonin variation (Snyder *et al.*, 1964; Fiske, 1964).

The psychotogenic piperidyl benzilates induce EEG synchrony and block EEG activation mediated by physostigmine (White and Carlton, 1963). It was postulated that these effects involved central anticholinergic mechanisms. Brimblecombe and Green (1968) concluded that JB336 blocked central cholinergic synapses involved in EEG arousal. Since maintenance of the pineal serotonin rhythm is mediated by sympathetic innervation from the central nervous system *via* the superior cervical ganglia, it is possible that blockade of cholinergic synapses may alter transmission of nerve impulses to the pineal. The circadian variation of plasma 17-hydroxycorticosteroid is abolished by atropine (Krieger and Krieger, 1967). The inference is that alterations in synaptic transmission in hypothalamic pathways occur after atropine.

Inhibition of RNA synthesis by actinomycin D reduced the noontime peak of pineal serotonin (Snyder *et al.*, 1967). It was suggested that the increase of serotonin from the midnight low may involve synthesis of RNA. Since puromycin had no effect on pineal serotonin, it appears that protein synthesis is not involved in maintenance of the serotonin rhythm. How the diurnal variation of pineal RNA and protein is linked to the rhythm of serotonin is uncertain, but it appears that all these variations are under nervous control that is modified by administration of JB336.

Since there are no data available on the kinetics of JB336 distribution or metabolism in the pineal, the absence of a drug effect on some of the parameters during the dark period (*e.g.*, serotonin content at 2400 hours and 0600 hours) may simply reflect an absence of the drug in the pineal.

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