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DIFFERENTIAL EFFECT OF BENSERAZIDE (Ro4-4602) ON THE CONCENTRATION OF INDOLEAMINES IN RAT PINEAL AND HYPOTHALAMUS

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1 Low doses (50 and 80 mg/kg) of benserazide (Ro4-4602), an aromatic amino acid decarboxylase inhibitor, markedly reduced 5-hydroxytryptamine and melatonin in the rat pineal gland without affecting hypothalamic 5-hydroxytryptamine.

2 This differential effect shows that inhibition of the pineal gland decarboxylase activity is possible, and confirms that the rat pineal gland is accessible to peripherally acting agents.

Introduction

Assessment of the physiological role of the indoleamine melatonin (MT) is not easy; few methods exist for suppressing its synthesis, or removing it from the circulation. The efficacy of pinealectomy as a method for completely removing MT from the circulation is controversial, since possible alternative sites of MT production exist in the gut (Raikhlin, Kvetnoy & Tolkachev, 1975), the retina and the Harderian gland (Cardinali & Wurtman, 1972; Bubenik, Purtill, Brown & Grota, 1978). Furthermore, physiological effects observed following pinealectomy may not be linked exclusively to any specific pineal compound, or group of compounds produced in the pineal gland. Immunisation against MT should reduce the availability of the free circulating compound (Knigge & Sheridan, 1976) but to date, detailed investigations concerning the amount of both free and bound circulating MT in immunised animals have not been undertaken. Propanolol reportedly decreases circulating MT in man (Hanssen, Heyden, Sundberg & Wetterberg, 1977), and causes an increase in 5-hydroxytryptamine (5-HT) at night in rats (Brownstein, Holz & Axelrod, 1973); however, this compound is a non-selective β-receptor blocker, giving peripheral and central effects.

It would clearly be advantageous, in the study of pineal function, to be able to inhibit pineal MT synthesis without affecting amine metabolism in the central nervous system. We show here that it is possible, by use of the aromatic amino acid decarboxylase inhibitor benserazide (seryl-trihydroxybenzyl-hydrazine; Ro4-4602) to inhibit the pineal production of MT and 5-HT without affecting hypothalamic 5-HT.

Methods

The animals used in these experiments were male albino Wistar rats (Porton strain) weight range 155–240 g, kept in a light:dark (L:D) schedule of 12 h:12 h and provided with Spratt's Rodent Laboratory Diet No. 1 and water *ad libitum*. Benserazide was a gift from Hoffman La Roche Ltd. Melatonin and 5-HT were obtained from the Sigma Chemical Co. Ltd. [^{3}H]-melatonin (26 Ci/mM) was obtained from New England Nuclear Corporation Ltd. All other reagents were commercially available and were analytical grade.

Experimental design

Animals were placed in a 12:12 L:D schedule one week before the experimental day. On this day they were divided into three groups (30 or 36 animals in each group) which received either (a) benserazide, 50 mg/kg, given intraperitoneally in 0.16 to 0.75 ml 0.9% saline; (b) benserazide, 80 mg/kg, given intraperitoneally in 0.16 to 0.75 ml 0.9% saline; or (c) an equivalent volume of 0.9% saline (controls). Five or 6 animals from each group were injected and killed at different times after lights on as follows: injected at 3.0 h, killed at 4.0 h; injected at 3.0 and 5.5 h, killed at 6.0 h; injected at 3.0, 5.5 and 8.5 h, killed at 9.0 h; injected at 3.0, 5.5, 8.5 and 11.5 h, killed at 12.0 h; injected at 3.0, 5.5, 8.5, 11.5 and 16.5 h, killed at 17.0 h; injected at 3.0, 5.5, 8.5, 11.5, 16.5 and 19.5 h, killed at 20.0 h. This experimental protocol is shown schematically in Table 1. With the exception of those animals killed at 4.0 h after lights on, multiple injections were

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chosen in order to achieve continuous inhibition of decarboxylase activity throughout the day and night. Times of killing were chosen to include the reported times of peak 5-HT and melatonin content of the rat pineal gland (Quay, 1963a; Wilkinson, Arendt, Bradtke & de Ziegler, 1977). During the dark phase animals were maintained and killed in dim red light. All experiments were performed in October 1979.

Experiment I: 6 animals from each group were killed by decapitation at intervals throughout the day and night. Pineals were dissected out and frozen within 30 s of death, stored at −20°C and later assayed for MT.

Experiment II: 5 animals from each group were killed by decapitation and the brains removed. The pineal glands were homogenized immediately in 200 ml of 0.01 M HCl and 2 ml of acidified butanol (0.86 ml conc. HCl in 1 litre of *n*-butanol), while the hypothalami were dissected out (Quay, 1963b), weighed, frozen and stored in liquid nitrogen and homogenized as described above within 30 min. The homogenates were then stored at 4°C until analysis for 5-HT within 48 h.

Assays

Pineal MT was determined by a previously described radioimmunoassay method (Arendt, Wetterberg, Heyden, Sizonenko & Paunier, 1977). All determinations were performed in the same assay. Intra-assay variability was 7.8%, 11.1% and 16.4% at 45, 93 and 15 pg respectively with this method.

The 5-HT assays were performed by a modification of the method of Snyder, Axelrod & Zweig (1965): 1.0 ml of the homogenate was washed twice with 0.1 ml of KCl saturated 2 M phosphate buffer pH 10. Then 0.8 ml of the homogenate was transferred to a centrifuge tube containing 2 ml of cyclohexane and 1 ml of 0.1 M HCl. 5-HT was returned to the aqueous phase by shaking the tubes. After centrifugation, the organic phase was aspirated and 0.8 ml of the aqueous phase was added to another tube containing 0.4 ml of 0.625 M $NaHPO_4$ and 0.1 ml of 0.1 M ninhydrin. The tubes were incubated at 70°C for 30 min. Sixty minutes after incubation, fluorescence of the samples was determined in a (Perkin Elmer MPF 3) spectrophotofluorometer (excitation 385 nm; emission 490 nm, both uncorrected).

Results

The results of the experiments are summarised in Figures 1, 2 and 3. Pineal 5-HT content (Figure 1) of the control rats showed the well-known circadian rhythm (Quay, 1963a) with high light-phase levels. The difference between the day time and night time levels is statistically significant ($P < 0.01$). There was a 60 to 90% reduction of pineal 5-HT content in rats injected with benserazide which appeared to be dose-related. The pineal 5-HT circadian rhythm of the drug-injected rats was abolished. The higher 5-HT level of the drug-injected rats killed at 6 h when compared to those killed at 4 h or 9 h after lights on could be due to the fact that the former group (in error) did not receive a benserazide injection 0.5 h before they were killed.

In the case of hypothalamic 5-HT content (Figure 2) there were no significant differences between control and drug-injected rats at any of the times examined. We did not demonstrate the circadian rhythm of hypothalamic 5-HT (Quay, 1968); however, the levels of 1 to 2 ng/mg were similar to those previously found (Quay, 1968; Anton-Tay, Chou, Anton & Wurtman, 1968). Figure 2 shows that hypothalamic 5-HT content was not affected by benserazide at the doses given.

Pineal MT in control animals showed the expected low light-phase values (Figure 3). A highly significant increase was seen in the dark phase, 20 h after lights on. Values are in the same range as those previously reported (Ozaki & Lynch, 1976; Pang, Brown, Grota,

Table 1 Experimental design

*Times of injections** (h after lights on)						*Times of death* (h after lights on)	*Total no. of injections*
3.0						4.0	1
3.0	5.5***					6.0***	2
3.0	5.5	8.5				9.0	3
3.0	5.5	8.5	11.5			12.0	4
3.0	5.5	8.5	11.5	16.5**		17.0**	5
3.0	5.5	8.5	11.5	16.5**	19.5**	20.0**	6

*Benserazide (50 mg or 80 mg/kg) or vehicle, see text for details; **injected and killed under dim red light; ***booster injection 30 min before rats were killed was not given in 5-HT study only.

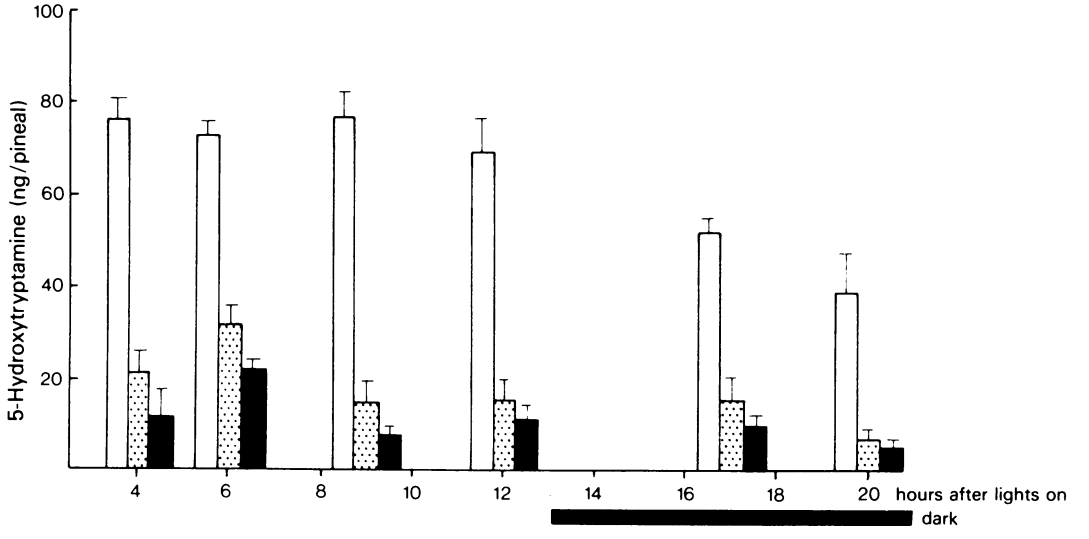

Figure 1 Pineal 5-hydroxytryptamine (5-HT) content (n = 5) in benserazide-treated and control rats at various times in the light and dark phase; vertical lines show s.e. mean. Open columns: saline-treated; stippled columns: benserazide 50 mg/kg; solid columns: benserazide 80 mg/kg. Differences between controls and benserazide-treated rats are significant ($P < 0.0002$ – <0.001, Student's t test, unpaired).

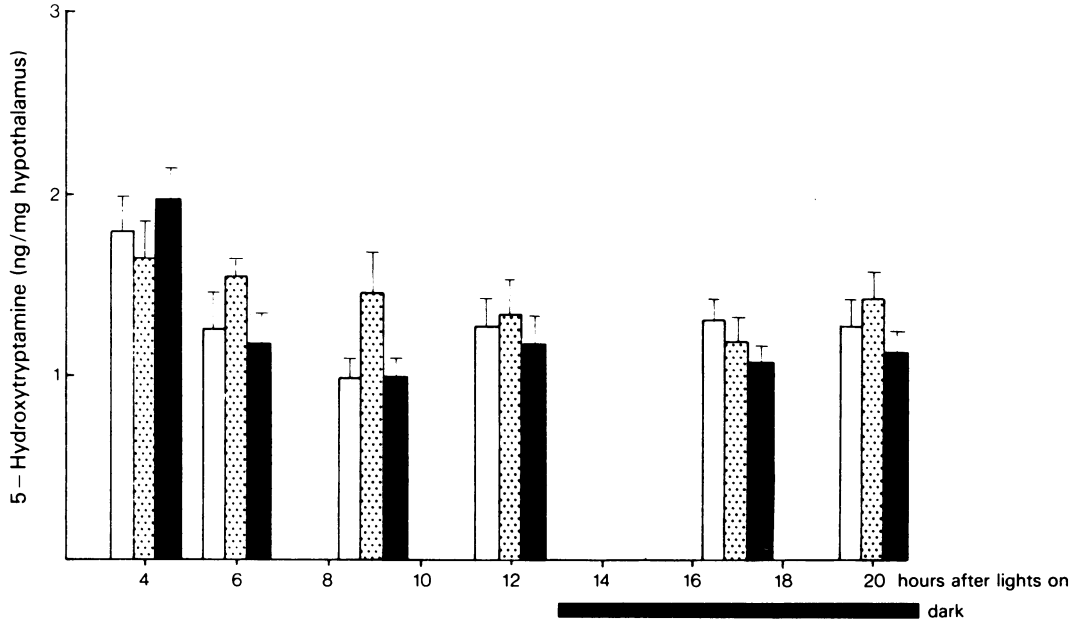

Figure 2 Hypothalamic 5-hydroxytryptamine (5-HT) content (n = 5) in benserazide-treated and control rats at various times in the light and dark phase; vertical lines show s.e. mean. Open columns: saline treated; stippled columns: benserazide 50 mg/kg; solid columns: benserazide 80 mg/kg. Differences between control and benserazide-treated rats are not significant (P = 0.1–0.68. Student's t test, unpaired).

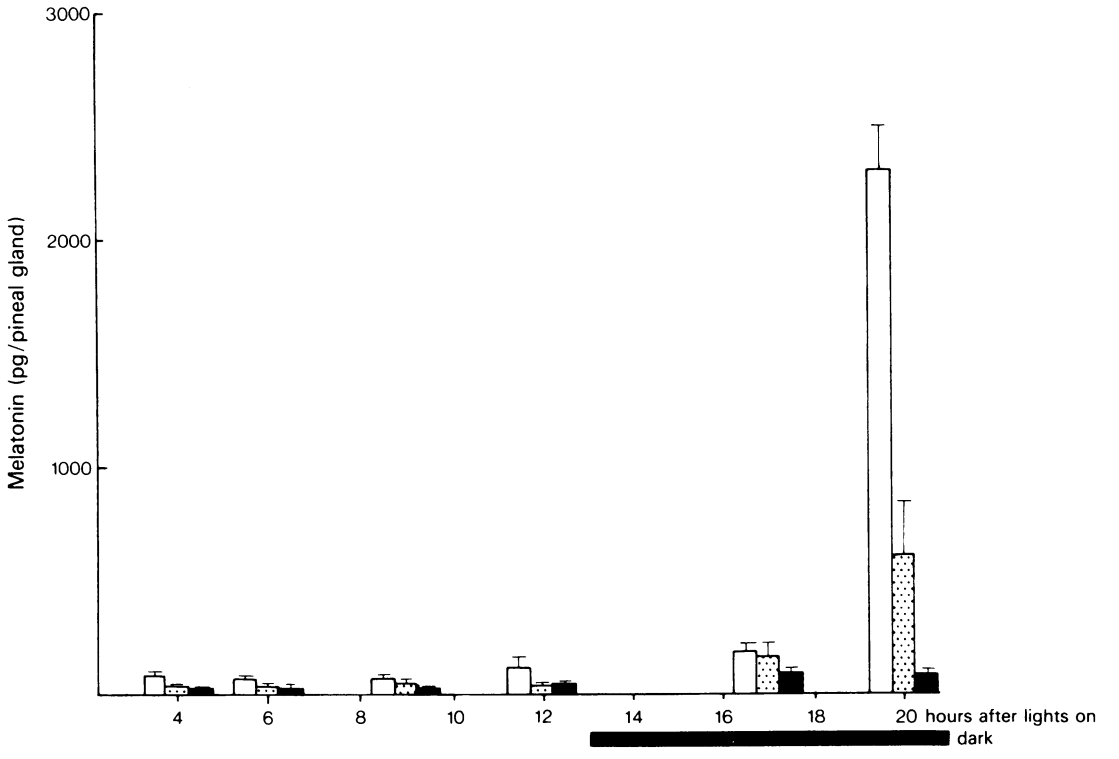

Figure 3 Pineal melatonin (MT) content (n = 6) in benserazide-treated and control rats at various times in the light and dark phase. Differences between control (open columns) and benserazide-treated rats at 80 mg/kg (solid columns) are significant ($P < 0.001$–0.05); at 50 mg/kg (stippled columns) are significant ($P < 0.001$–0.01) except for 9 and 17 h after lights on. Significance levels were assessed by Student's t test, unpaired.

Chambers & Rodman, 1976; Wilkinson *et al.*, 1977). Benserazide (50 mg/kg) significantly reduced pineal MT content at all time points except 9 and 17 h after lights on. Benserazide (80 mg/kg) significantly reduced pineal melatonin at all time points, the reduction being 97% of control levels at the 20 h time point.

Discussion

Preliminary experiments with benserazide (Symons, Laxton & Arendt, 1979) indicated that centrally acting doses of this compound (500 mg/kg) greatly reduced dark phase pineal MT content (to 5% of control values) and that a low dose (50 mg/kg) considered to act peripherally (Bartholini, Burkard, Pletscher & Bates, 1967), reduced dark phase pineal MT content to 41% of control values.

These experiments were undertaken in order to determine whether it was possible to suppress completely pineal MT and 5-HT synthesis without affecting the central nervous system. Hypothalamic 5-HT content was used as an index of benserazide penetration into the CNS.

Administration of benserazide (80 mg/kg) with the injection schedule used, clearly greatly reduced pineal MT and 5-HT content without affecting the 5-HT content of the hypothalamus. While it is conceivable that other parts of the CNS may have been affected by this dose of benserazide this seems unlikely particularly as the hypothalamus is considered to have an incomplete blood-brain barrier (Clementi & Ciccarelli, 1970).

The observation that benserazide reduced pineal 5-HT and MT content may be explained by direct inhibition of 5-hydroxytryptophan decarboxylation, so limiting 5-HT production, together with a reduction in catecholamine production by dopa decarboxylase inhibition (Lovenberg, Weissbach & Udenfriend, 1961). Thus in so far as MT is concerned, both production of its precursor (5-HT) and noradrenergic stimulation of 5-HT N-acetyl-transferase activity (5-HT-NAT), normally rate-limiting in MT synthesis,

are likely to be affected. The effect of benserazide on 5-HT-NAT is now under investigation.

On comparison of the reduction of the 5-HT content with the MT content of the pineal, it seems that a residual 5-HT content was maintained at the doses employed. This may be attributed to a reduction of parenchymal 5-HT with little effect on neuronal 5-HT (Falck, Owman & Rosengren, 1966). This differentiation in the location of pineal 5-HT was reported by Bertler, Falck & Owman (1964). Higher doses of benserazide, or a longer treatment period may reduce pineal 5-HT levels even further.

There is an existing doubt as to what extent the pineal is within or without the blood brain barrier (Wurtman, Axelrod & Kelly, 1968). Silver nitrate and other dyes have been used to demonstrate the less restrictive barrier system of the pineal (Dempsey & Wislocki, 1955) but such leakage of dyes or particles could be limited to the perivascular connective tissue compartments rather than the parenchyma itself. These results confirm that, in the rat, the pineal is accessible to peripherally acting agents, i.e. is pharmacologically without the blood-brain barrier. Doubt concerning the accessibility of the human pineal gland could be resolved by use of a similar experimental approach in volunteers.

Injection of MT can lead to an increase in 5-HT content of the hypothalamus (Anton-Tay *et al.*, 1968). On the other hand, pinealectomy causes a decrease in endogenous 5-HT in the hypothalamus (Moszkowska, Kordon & Ebels, 1971; Sugden & Morris, 1979). However, in the present experiments the 5-HT content of the hypothalamus remained more or less the same in spite of the reduction of MT in the pineal. This apparent contradiction could possibly be explained by experimental time differences in measuring hypothalamic 5-HT. Our results were obtained after hours of drug treatment, while after pinealectomy, an increase in 5-HT in the hypothalamus was seen after 10 days when the rats had recovered from the operation. This may suggest an advantage of chemical lesion over pinealectomy in that no recovery period is required. Any rapid changes in the central nervous system due to disturbance of pineal metabolism may be detected, before readjustment by the body. However, it should be borne in mind that the modification of peripheral aromatic amino acid metabolism can indirectly affect brain function (Fernstrom & Wurtman, 1971).

In conclusion we propose that benserazide, at 80 mg/kg, with its ability to interfere with pineal metabolism and its inability to pass through the blood brain barrier could be used as an inhibitor of indoleamine synthesis in the pineal. Should a situation arise where pathological amounts of pineal indoleamines are produced, peripheral decarboxylase inhibition may provide a therapeutic approach.

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