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# Synthesis of Serotonin by Pineal Glands of the Rat in Organ Culture

THE pineal gland of the rat has been shown to contain large quantities of the indole serotonin[1]. This organ can also metabolize serotonin by a unique pathway to form the hormone melatonin (5-methoxy *N*-acetyltryptamine)[2]. The content of serotonin[1,3] and the activity of the melatonin-forming enzyme[4] in the pineal have been found to vary with a 24 h cycle. These rhythms are controlled by information transmitted to the pineal through its sympathetic nerves[3,5].

Little is known about the biochemical control of serotonin synthesis in the intact pineal gland. The investigation of this and other regulatory processes in the pineal may be facilitated by the development of an *in vitro* system in which serotonin synthesis persists. Previous investigators have grown mammalian pineal glands in organ and tissue culture[6–10], and have shown that the cells which appear bear a histological resemblance to pineal parenchymal cells[8,9]. The biochemical properties of pineal cells in culture, however, have not been examined yet. The present communication describes a technique for cultivation of the pineal glands of adult rats, such that serotonin is synthesized from the amino-acids tryptophan and 5-hydroxytryptophan with a rate that increases during the first 4 days in culture. Additional investigations provide evidence for the continuing activity of both the enzymes required for serotonin synthesis (tryptophan hydroxylase and 5-hydroxytryptophan decarboxylase), and for a rate-limiting role of tryptophan hydroxylase in the formation of this amine.

Sprague–Dawley female rats weighing 160–180 g were killed by neck fracture, and the pineal glands were quickly removed[2,4] and transferred to a Petri dish, where they were covered with neutralized Hanks solution. The gland was then divided into two approximately equal parts. These fragments were clotted to the walls of a Wasserman tube (which had previously been coated with a thin film of chicken plasma) by the application of chick embryo extract to the glass surface[11]. Fifteen to thirty minutes later, when the clot was sufficiently firm, 0·5 ml. of nutrient medium was added to the culture; the tube was sealed with a rubber stopper and incubated in a roller wheel at 37° C. The nutrient medium consisted of 75 per cent Puck N 16 medium, 5 per cent Evans NCTC 109 medium, 10 per cent foetal calf serum and 10 per cent heat-inactivated (56 per cent C for 30 min) horse serum. The constituents of the medium were obtained as standard

preparations, except for the N 16 and NCTC 109, which were prepared without phenol red. (This indicator dye had been found in preliminary experiments to interfere with the assay for serotonin.) The nutrient medium as described contained tryptophan in a concentration of approximately $10^{-4}$ molar, but not 5-hydroxytryptophan. For certain experiments the medium was fortified with $10^{-3}$ molar tryptophan or $10^{-5}$–$10^{-3}$ molar 5-hydroxytryptophan. At 2 day intervals the media were poured off and stored at $-70°$ C for serotonin assay; new media were added, and the incubation was resumed. Each tube contained 25 U of penicillin, 25 μg of streptomycin, and 0·5 μg of amphotericin *B* ('Fungizone'). Each experimental group contained seven culture tubes.

All assays for serotonin were performed on the same day on 0·1 ml. aliquots of medium. The method of assay involved the formation of a fluorescent product after extracting the serotonin[12]. The identity of the serotonin was confirmed by thin-layer chromatography in an ethanol : dioxane : benzene : ammonia system (5 : 40 : 30 : 5), followed by staining with Ehrlich's reagent.

Cells in explanted pineal fragments had a healthy appearance throughout the 10 day period of observation. Examined in preparations stained with haematoxylin and eosin, the cell population after the first 2–3 days appeared to consist almost entirely of pinealocytes. There was little cellular outgrowth from the explanted fragments until the fourth day; however, by the eighth to tenth day an extensive growth appeared from all fragments. This outgrowth also consisted almost entirely of pinealocytes, with occasional scattered, more elongated cell forms. These morphological findings were similar to those described[9,10] in plasma clot cultures of rat pineal fragments, except that the outgrowth in the present investigation included fewer cells other than pinealocytes.

The net synthesis of serotonin by the pineals was determined by comparison of the quantity of amine released into the medium during 2 day periods of incubation with the amounts present in two kinds of control tubes: "zero-time" tubes, which were frozen immediately after the addition of the pineal, and tubes which were incubated without pineal tissue. When incubated with 0·5 ml. of medium containing $10^{-4}$ molar tryptophan, pineals were capable of synthesizing as much as 1·5 μg of serotonin in a day. (This amount is several times greater than the highest reported concentrations of serotonin in the rat pineal, but is consistent with the rapid rate at which the serotonin content of the gland normally increases *in vivo* early in the day[1]). The medium did not contain 5-hydroxytryptophan, and so the synthesis of serotonin in these conditions indicated that the pineal gland contained both the 5-hydroxylating and the decarboxylating enzymes (that is, tryptophan hydroxylase

and 5-hydroxytryptophan decarboxylase). The rat pineal has previously been shown to contain relatively large amounts of this latter enzyme[12]. Tryptophan hydroxylase activity has already been found in brain[14], but not so far in the pineal.

To determine which of these two enzymes controlled the rate of serotonin synthesis by pineals in organ culture, glands were incubated with media fortified by the addition of tryptophan or 5-hydroxytryptophan (Table 1). Pineals incubated with $10^{-4}$ molar tryptophan generated $4{\cdot}3 \times 10^{-9}$ moles of the amine during the first 2 days of culture. (This represented an 8·5 per cent conversion of the amino-acid.) Increasing the concentration of the tryptophan ten-fold (to $10^{-3}$ molar) did not increase the absolute yield of serotonin. Pineals incubated with $10^{-5}$ molar 5-hydroxytryptophan generated $3{\cdot}8 \times 10^{-9}$ moles of the amine in 2 days. When a greater concentration of this amino-acid was used, there was a marked rise in the rate of serotonin synthesis (Table 1). These data indicated that the activity of the decarboxylase enzyme was considerably greater than that of the hydroxylating enzyme. They further suggested that, in the conditions of this experiment, the hydroxylation step determined the rate at which serotonin was formed.

Table 1. SEROTONIN SYNTHESIS BY RAT PINEAL GLANDS IN ORGAN CULTURE

| Contents of medium | Moles of serotonin formed ($\times 10^{-9}$)/tube Zero time | 0–2 days | 2–4 days |
|---|---|---|---|
| Tryptophan | | | |
| $10^{-4}$ molar | 1·0 ± 0·1 | 4·3 ± 0·3* | 1·6 ± 0·2† |
| $10^{-3}$ molar | 0·9 ± 0·2 | 4·3 ± 0·4* | 3·8 ± 0·9† |
| $10^{-4}$ molar, no pineal | 0·9 ± 0·1 | 0·9 ± 0·1 | 0·8 ± 0·1 |
| 5-Hydroxytryptophan (also contained $10^{-4}$ molar tryptophan) | | | |
| $10^{-5}$ molar | 1·1 ± 0·2 | 3·8 ± 0·4* | 2·8 ± 0·3* |
| $10^{-4}$ molar | 1·6 ± 0·3 | 6·5 ± 0·6* | 8·1 ± 0·6* |
| $10^{-3}$ molar | 1·6 ± 0·3 | 9·9 ± 0·6* | 10·9 ± 0·8* |
| $10^{-3}$ molar, no pineal | 1·4 ± 0·1 | 1·5 ± 0·2 | 1·4 ± 0·1 |

Groups of seven culture tubes, each containing one pineal gland, were incubated for 2 days. The medium was then poured off and frozen, fresh medium was added, and the tubes were incubated for 2 more days. Control tubes were frozen immediately after the addition of the pineal, or were incubated without a pineal. Aliquots of the culture medium (0·1 ml.) were assayed for serotonin content.

*$P < 0{\cdot}001$, differs from zero time controls.

†$P < 0{\cdot}01$, differs from zero time controls.

In all experiments, the synthesis of serotonin from 5-hydroxytryptophan was greatest on days 2–4. Significant, but decreasing, amounts of the amine continued to be formed for 8–10 days after initiation of the culture. The increase in decarboxylase activity between days 0–2 and 2–4 suggests that new enzyme is being formed in organ culture. This could represent more enzyme in each pineal cell, or unchanged levels in a larger number of cells.

Previous work has shown that the activity of 5-hydroxytryptophan decarboxylase in the rat pinealocyte is regulated by the sympathetic nerves to this organ[13]. It is presumed that the sympathetic nerve ending exerts its effects by release of a neurotransmitter substance. The

development of an *in vitro* system which continues to synthesize serotonin for several days could provide a useful experimental tool for investigation of the neural control of pineal enzymes.

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