Biochimica et Biophysica Acta, 526 (1978) 93—99

BBA 68534

N-ACETYLTRANSFERASE ACTIVITY OF THE RAT HARDERIAN GLAND

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(Received January 19th, 1978)
(Revised manuscript received May 19th, 1978)

Summary

Harderian gland extracts from male rats catalyze the conversion of serotonin to *N*-acetylserotonin and of tryptamine to *N*-acetyltryptamine.

The reaction is linear up to 14 mg tissue and departs from linearity after 10 min. The pH optimum with tryptamine as substrate is between 8 and 9.

Enzymic activity of the gland in vivo does not show diurnal variations.

Enzymic activity of tissue in organ culture is not stimulated by 10 μM isoproterenol or 100 μM dibutyryl cyclic AMP.

Harderian gland tissue in culture can acetylate tryptamine and serotonin and can O-methylate the *N*-acetylserotonin to form melatonin.

Introduction

The Harderian gland was first identified by the Swiss anatomist Harder [1] in 1694 and has a wide phylogenetic distribution. In the rat, the gland fills the medial and posterior section of the orbit and surrounds the optic nerve. Its function is not known. The gland is rich in porphyrins [2] and some evidence suggests that it may affect indole metabolism in the infant rat pineal [3,4]. The gland is reported to have a hydroxyindole-*O*-methyltransferase (*S*-adenosyl-L-methionine:*N*-acetylserotonin *O*-methyltransferase, EC 2.1.1.4) capable of catalyzing the synthesis of melatonin from *N*-acetylserotonin [5], but the activity differs from the enzyme in the pineal gland or retina in being Mg^{2+}-dependent and in having a lesser affinity for *N*-acetylserotonin. Immunohistochemical studies [6,7] have indicated that the Harderian gland contains melatonin in its secretory cells. However, it is not known whether *N*-acetylserotonin can be made by the gland in situ or whether it must be supplied by other tissues.

Although an inducible form of *N*-acetyltransferase (acetyl-CoA:arylamine *N*-acetyltransferase, EC 2.3.1.5), which catalyzes the acetylation of serotonin, seems localized in the pineal gland, a non-inducible enzyme catalyzing this reaction is reported to occur in several extrapineal tissues [8]. In this paper we have examined the Harderian gland for *N*-acetyltransferase activity.

Methods and Materials

Tissue. Harderian glands were obtained from 100—120 g male Sprague-Dawley rats bred in the laboratory from Charles River stock and maintained on a 12 h : 12 h light-dark cycle in a temperature-controlled room. Animals were killed between 09.00 and 10.00 in all but the diurnal variation studies.

Chemicals. Acetyl-CoA and tryptamine were obtained from Schwartz/Mann (Orangeburg, N.Y.), melatonin from Aldrich (Milwaukee, Wisc.), serotonin creatinine sulfate from Sigma (St. Louis, Mo.), hydroxytryptophol, methoxytryptophol, methoxyindoleacetic acid, and hydroxyindoleacetic acid from Regis (Morton Grove, Ill.), L-tryptophan from Calbiochem (La Jolla, Calif.), [^{14}C]acetyl-CoA (51.5 Ci/mol) and L-[G-^{3}H]tryptophan (7.3 Ci/mmol) from New England Nuclear (Boston, Mass.), and 5-hydroxy[side chain-2-^{14}C]tryptamine creatinine sulfate (56 Ci/mol) from Amersham/Searle (Arlington Heights, Ill.).

Assays. Harderian glands were minced and suspended in 3 vols. (v/w) ice-cold 0.1 M sodium phosphate buffer (pH 6.8). The suspension was sonicated (Branson LS75, 3 A, 5 s), or homogenized in a Teflon homogenizer, centrifuged at 2000 $\times g$ for 10 min at 5°C, and the brick red suspension (between the lipid layer and the precipitate) drawn off for enzymatic assay. *N*-Acetyltransferase activity was assayed principally by the method of Deguchi and Axelrod [9] as modified by Parfitt et al. [10]; tryptamine and [^{14}C]acetyl-CoA were the added substrates and the reaction was terminated and labeled product extracted by the addition of $CHCl_3$. *N*-acetyltransferase activity was also assayed by two other methods. In a method modified from Weissbach et al. [11], incubation conditions were the same as for the Deguchi and Axelrod method except that serotonin was substituted for tryptamine. Termination of the incubation and isoamyl alcohol extraction of the labeled products were as described by Weissbach et al. [11]. In an additional assay modified from Klein et al. [12], tissue supernatant was added to a reaction mixture with final concentrations of 0.4 mM acetyl-CoA, 0.7 mM 5-hydroxy[*side chain*-2-^{14}C]tryptamine creatinine sulfate (55 Ci/mol) and 0.1 M sodium phosphate (pH 6.8) in a final volume of 0.1 ml. The reaction was terminated by addition of 60 μl 50% ethanol/0.5 M HCl containing 3 mg ascorbic acid and 10 μg each of *N*-acetylserotonin and melatonin. Following chromatographic separation, the areas corresponding to *N*-acetylserotonin and melatonin were scraped into scintillation vials containing 0.5 ml ethanol, shaken for 10 min and suspended in 4.2 ml water and 10 ml Instagel (Packard, Chicago, Ill.) for radioactivity determination.

Tissue cultures. Pieces of Harderian gland or pineal glands were cultured by a modification of the methods of Strada et al. [13] and Parfitt et al. [14]. Medium BGJb (Fitton-Jackson modification) containing 100 units/ml penicillin,

50 μg/ml streptomycine and 1 mg/ml bovine serum albumin (but without ascorbic acid, glutamine, or phenol red) was purchased (Grand Island Biological, Grand Island, N.Y.). Just before use, sodium ascorbate and glutamine were added to concentrations of 0.5 and 2 mM, respectively. Harderian glands were minced into approx. 1-mm cubes and two such cubes were placed on a circular nylon mesh support screen in each well. Drugs were added in 10-μl volumes of either 0.001 M HCl or water. At the end of the incubation period the glands and the medium were separately frozen in assay tubes on solid CO_2. In some instances *N*-acetyltransferase activity of the tissue was determined by the method of Deguchi and Axelrod [9] as described above. In instances of incubation in the presence of radioactively labeled precursors, labeled indoles in the gland and in the medium were determined by chromatography and scintillation counting of chloroform- or isoamyl alcohol-extractable materials. Proteins were determined by the method of Lowry as described by Layne [15].

Results

Harderian gland extract was incubated with [^{14}C]acetyl-CoA and tryptamine according to the method of Deguchi and Axelrod [9]. Following the addition of $CHCl_3$, CH_3OH was also added to yield final proportions of $CHCl_3/CH_3OH$/incubation medium of 2 : 1 : 0.6 (v/v). A concentrate of the non-polar phase was separated by thin-layer chromatography on silica gel. Development twice in heptane/diethyl ether/acetic acid (80 : 19 : 1, v/v) to move neutral lipids to the solvent front was followed by development in $CHCl_3/CH_3OH$/acetic acid (82 : 7 : 1, v/v). Of the applied label, 93% was found to be isochromatographic with melatonin (melatonin and *N*-acetyltryptamine are isochromatographic under these conditions). Only 7% of the label was associated with the neutral lipid fraction. From these results it was concluded that Harderian gland extracts catalyze the acetylation of tryptamine.

In similar experiments, Harderian gland extract was incubated with [^{14}C]-acetyl-CoA and serotonin. Solvent partitioning and thin-layer chromatography were performed as described above. Of the applied radiolabel, 72% was found in the neutral lipid fraction, 23% was isochromatographic with *N*-acetylserotonin, and 2% was isochromatographic with melatonin. Further, similar incubations containing serotonin were terminated by the solvent partitioning method of Weissbach et al. [11]. The resulting isoamyl alcohol extract was concentrated and separated by thin-layer chromatography as described by Klein et al. [12]. Of the applied radiolabel, 29% was found to be isochromatographic with *N*-acetylserotonin and about 3% isochromatographic with melatonin. From these results it was concluded that Harderian gland extracts catalyze the acetylation of serotonin.

The results of assays of *N*-acetyltransferase activity using either tryptamine or serotonin as substrate and the effects of heating the tissue extract are shown in Table I.

Enzyme properties

The relationship between tissue concentration and the extent of reaction was determined by the method of Deguchi and Axelrod [9] using tryptamine as

TABLE I

ACETYLATION OF TRYPTAMINE AND SEROTONIN

The Harderian glands (640 mg) from three rats were homogenized in 1.9 ml 0.1 M sodium phosphate buffer (pH 6.8), and the homogenate centrifuged at 2500 × *g* for 10 min. 20 μl supernatant was used in each assay. In some samples the supernatant/buffer mixture was heated at 70°C for 10 min before addition of other materials. When tryptamine was used as substrate, the reaction was assayed by a modification of the method of Deguchi and Axelrod [9]. When serotonin was used as substrate, the reaction was assayed by a modification of the method of Weissbach et al. [11]. Values reported are the mean of three determinations ± S.E.

	Enzyme activity (nmol/h per 20 μl)	
	Tryptamine	Serotonin
Complete assay	2.09 ± 0.11	3.01 ± 0.18
Minus substrate	0.31 ± 0.04	1.15 ± 0.03
Minus supernatant	0.09 ± 0.01	0.55 ± 0.02
Heated supernatant	0.37 ± 0.05	0.73 ± 0.04

substrate and by the methods of Weissbach et al. [11] and Klein et al. [12] using serotonin as substrate. As can be seen in Fig. 1, the reaction was linear with tissue in all three assay systems. However, considerable radioactivity was extractable into isoamyl alcohol in the Weissbach assay even when serotonin was omitted from the system. This is in accord with the results in Table I, and likely reflects endogeneous substrates in the tissue extract. The reaction was linear with time for the first 10 min, but departed from linearity thereafter (Fig. 2). The pH-dependence of the reaction is shown in Fig. 3. In the absence

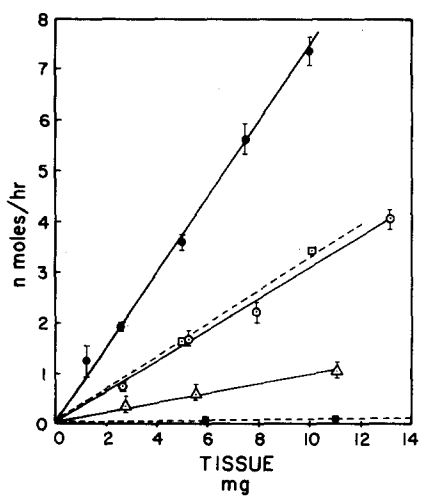
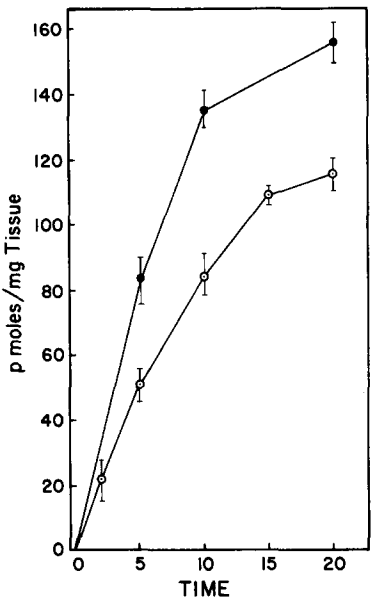


Fig. 1. Relationship between rate of acetylation and tissue. Supernatant solutions from Harderian gland sonicates were assayed by the method of Deguchi and Axelrod [9] when tryptamine was substrate (open circles) and by the method of Weissbach et al. [11] (closed circles) or the chromatographic procedures of Klein et al. [12] (open triangles) when serotonin was substrate. The dotted line and open squares show the accumulation of radioactive products in the Weissbach assay in the absence of exogenous substrate. The dotted line and closed squares show the accumulation of radioactive products in the Deguchi and Axelrod assay when substrate was omitted. Each point is the mean for three or more samples. The bars represent standard errors of the mean.



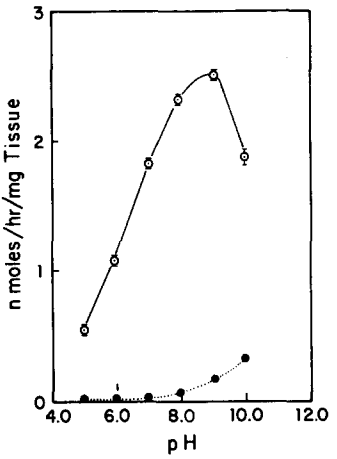


Fig. 2. Acetylation of serotonin or tryptamine with time. The Deguchi and Axelrod assay was used to determine the extent of acetylation when tryptamine was substrate (open circles) and that of Weissbach et al. [11] was used when serotonin was substrate (close circles). Each point is the mean for triplicate samples. The bars represent standard errors of the mean.

Fig. 3. pH dependence of acetylation. Harderian glands were sonicated in saline, centrifuged, and 30 μl added to the reaction mixture described by Deguchi and Axelrod (cf. Methods and Materials) to give a final volume of 0.1 ml which was 0.1 M in phosphate (pH 5—8) or Tris (pH 9—10) buffer. The open circles are the values obtained after subtraction of values for blanks not containing enzyme (closed circles, dotted line). Each value is the mean for three determinations. The bars represent the standard error of the mean.

of enzyme, extractable radioactivity increased 10-fold between pH 6 and 10. The pH optimum for the *N*-acetyltransferase lies in the range pH 8—9. This is in the same range as that reported for the pineal daytime enzyme [16] and contrasts with the pH optimum of 6.5 reported for the dark-activated pineal enzyme [9]. It is higher than the 7.5—8.5 reported for amine-acetylating enzyme in liver [10] and brain [17].

Diurnal variation

The activity of the *N*-acetyltransferase of the pineal gland shows very marked circadian variations. In order to determine whether this enzyme in the Harderian gland varied similarly, animals were killed at 09.00, 16.00, and 24.00. The Harderian glands were quickly removed, frozen in solid CO_2, and stored at −70°C until assayed. Enzyme activities assayed after sonication were, respectively, 14.3 ± 0.1, 15.1 ± 0.6, and 14.0 ± 0.2 nmol/h per mg protein (mean ± S.E.). Thus there was no indication of circadian variations in this enzyme. The enzyme therefore resembles that described by Ellison et al. [8] in several extrapineal tissues, although the activity we observe in Harderian gland is slightly higher than the levels reported for other extrapineal tissues.

TABLE II

EFFECT OF ISOPROTERENOL AND DIBUTYRYL CYCLIC AMP ON *N*-ACETYLTRANSFERASE ACTIVITY OF HARDERIAN GLAND AND PINEALS IN ORGAN CULTURE

Pineal glands and approx. 1-mm cubes of Harderian gland tissue were cultured for 5 h in the presence or absence of additives. At the end of incubation, tissues were removed and frozen in tubes on solid CO_2. The frozen tissue was subsequently assayed for *N*-acetyltransferase acitvity by the method of Deguchi and Axelrod [9]. Each value is the mean for 3—6 determinations.

Additions	*N*-Acetyltransferase activity	
	Harderian gland (nmol/h per mg tissue)	Pineal (nmol/h per pineal)
None	0.21	0.36
Isoproterenol (20 μM)	0.20	4.06
Dibutyryl cyclic AMP (100 μM)	0.21	not determined

Tissue cultures

The data in Table II show the effect on *N*-acetyltransferase activity of additions of dibutyryl cyclic AMP or isoproterenol to acutely cultured Harderian gland. Activity of the enzyme was unaffected by either of these agents in marked contrast to their activation of the pineal enzyme. Enzymic activity in Harderian glands cultured for 48 h was barely above blank values and was unaffected by 4 h exposure to 100 μM norepinephrine, γ-aminobutyric acid, or acetylcholine.

The ability of the Harderian gland to convert tryptophan and serotonin to *N*-acetylated derivatives in vitro was determined by culturing Harderian gland with radiolabeled tryptophan (280 μM, 3.8 Ci/mol) or serotonin (20 μM, 56 Ci/mol) for 5 h. Tissue and media were separately analyzed for the formation of chloroform-soluble radioactive products. *N*-Acetylserotonin constituted 1.5% and melatonin 1.8% of the chloroform-extractable radioactivity in media from tissue cultured in the presence of radiolabeled serotonin. The bulk of identifiable indolic label (21%) was in 5-hydroxytryptophol. 5-Hydroxyindoleacetic acid is not extractable under these conditions. More importantly, labeled *N*-acetylserotonin and melatonin were also identified in the media from tissue cultured in the presence of labeled tryptophan. The yield, as might be expected, was lower (0.4% for *N*-acetylserotonin and 0.2% for melatonin) with the bulk of identifiable indolic radioactivity being in methoxytryptophol (9%). This suggests that this tissue has the capacity to hydroxylate tryptophan although this appears to be a relatively minor pathway of tryptophan metabolism.

Discussion

Harderian gland tissue has been reported to contain N-acetylated indoles [6,7] and a magnesium-dependent hydroxyindole-*O*-methyltransferase [5]. The present results demonstrate that the gland in vitro is capable of synthesizing serotonin from exogenous tryptophan and *N*-acetylserotonin from serotonin. However, the bulk of indole metabolism appears to be via oxidation. This is indicated by the preponderance of identifiable counts in indolic alcohols

extractable into chloroform from the culture media. The acidic catabolites are not extractable into chloroform under these conditions and, in most tissues, these constitute the predominant oxidized catabolites.

The activity of the enzyme responsible for the N-acetylation of tryptamine or serotonin appears to be non-inducible in organ culture and unresponsive to circadian changes in vivo. It thus appears similar to the generalized non-inducible *N*-acetyltransferase found in a number of extrapineal tissues. Further, the pH optimum of the Harderian gland enzyme is similar to that reported for non-induced pineal gland [16] and extra-pineal tissues [16,17], but is higher than the pH optimum reported for induced pineal gland enzyme [9]. As a minor note of interest, sonication of Harderian gland yields a higher specific activity of *N*-acetyltransferase than does homogenation of the gland. This again is similar to the pineal gland. The hydroxy-*O*-methyltransferase reported in this gland appears only minimally affected, if at all, by alterations is external lighting [5]. While the function of the Harderian gland is unknown, the existence in the gland and the characteristics of the enzymes required for formation of melatonin suggests that this tissue could provide the organism with a small but continuous supply of melatonin independently of the pineal. This may explain the continued presence of melatonin in rat hypothalamus [18] or in sheep plasma after pinealectomy [19] and may also account for the failure of pinealectomy to produce consistent endocrine changes in some species [20].

Acknowledgements

This study was supported in part by the U.S. Veterans Administration, U.S. Public Health Service grant RR05756 and grant 3371 from the Swedish Medical Research Council. The work was performed while Dr. Wetterberg was a visiting scientist at the Neurobiochemistry Laboratory. The authors are indebted to Ms. Selma Plotkin for technical assistance.

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