Identification of *O*-Acetyl-5-methoxytryptophol in the Pineal Gland by Gas Chromatography–Mass Spectrometry

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The presence of *O*-acetyl-5-methoxytryptophol in the pineal glands of rats kept in the dark for 8 h, but not in the light, has been shown by means of g.l.c.–mass spectrometry. It is suggested that this compound may be the biologically active precursor of circulating methoxytryptophol.

5-Methoxytryptophol exerts a powerful control over the gonads of both immature and mature animals. In chicks it stimulates both testicular and ovarian growth and development, whereas in the adult it shows anti-gonadal activity (Balemans, 1972, 1973; Balemans *et al.*, 1977*a,b*). Similarly in rats it inhibits oestrus (McIsaac *et al.*, 1964) and decreases spermatogenesis (Mas & Oaknin, 1977). It also prevents ovarian development caused by the administration of human choriogonadotropin to rats when given simultaneously (Vaughan *et al.*, 1976).

Smith *et al.* (1979) have shown that *O*-acetyl-5-methoxytryptophol (III) is synthesized in the pineal gland at the same time as melatonin [*N*-[2-(5-methoxytryptophol-3-yl)ethyl]acetamide; *N*-acetyl-5-methoxytryptamine, I} and methoxytryptophol

(II). This report was based on data from incubations of pineal glands with ³H-labelled *S*-adenosyl-methionine, a methyl donor, followed by t.l.c. of the products, when co-chromatography of synthetic *O*-acetyl-5-methoxytryptophol and radioactivity was observed in a number of solvents. They have also found that *O*-acetyl-5-methoxytryptophol is rapidly hydrolysed on incubation in human plasma (R. M. Leone & I. Smith, unpublished work). We have now applied g.l.c.–mass spectrometry to the problem in order to produce an absolute identification of the existence of *O*-acetyl-5-methoxytryptophol.

Materials and Methods

O-Acetyl-5-methoxytryptophol

This was synthesized by adding 3.5 ml of acetic anhydride to 10.5 ml of dry pyridine and 700 mg of 5-methoxytryptophol in a stoppered tube and leaving overnight. Most of the solvent was removed by rotary evaporation under vacuum and the residue was poured into water, when a precipitate of *O*-acetyl-5-methoxytryptophol appeared immediately. It was recrystallized from benzene/hexane (65:35, v/v) to constant m.p. (65.5–67°C). On t.l.c. it showed a single spot in ten different solvents, and after hydrolysis only 5-methoxytryptophol was found.

Tissues

Wistar rats, 160–200 g, from the Courtauld Institute colony were used throughout. Pineal and salivary glands, muscle, lung and adipose tissue were taken from 25 male and 25 female rats during

the day (18h light) and the night (6h darkness); the midday temperature was 22°C. Animals killed during the day had been exposed to at least 8h light. Animals killed during the night had been kept in the dark for 9h, and tissues were taken under weak red light (Ilford safety-light filter 906 ISO). All tissues were collected in a small volume of ice-cold 0.9% NaCl and stored at -20°C until used.

Extraction of *O*-acetyl-5-methoxytryptophol

All tissues were homogenized in 2 ml of redistilled chloroform, centrifuged at 400g for 10min and the aqueous phase and fat removed. The chloroform layer was pipetted into a vial and concentrated under a stream of argon. The concentrate was divided into four equal portions, transferred to tapered amber-glass microvials (Pye-Unicam), cooled in liquid N₂, placed in a vacuum desiccator containing silica gel and the chloroform removed *in vacuo*. Reagent blanks were carried through the whole procedure.

Production of derivatives for g.l.c.-mass spectrometry

Redistilled dry pyridine (5μl) plus 15μl of *N*-methyl-*N*-trimethylsilyltrifluoroacetamide (Pierce) were added to each vial, which was then capped by means of a hand crimper (Pierce) and heated for 5 min at 50°C. Portions (5μl) were injected into the g.l.c. column.

G.l.c.-mass spectrometry

The apparatus and conditions were as described by Leone *et al.* (1979a). Briefly, a glass column (1.5m×4mm) was packed with 10% OV1 on Chromasorb WHP (80–100 mesh) and g.l.c. was carried out isothermally with an oven temperature of 250°C and a helium-carrier-gas flow of 70ml/min. A Varian 311A double-focusing mass spectrometer was used with a Biemann-Watson two-stage separator at 280°C. Total-ion-current measurements at 70eV gave electron impact spectra that were recorded on u.v.-sensitive paper; background spectra were subtracted manually and the remaining spectra were normalized. Single-ion-detection measurements were made at 245, 232 and 305 *m/e* at a resolution of 4000.

Under these conditions, synthetic *O*-acetyl-5-methoxytryptophol gave a retention time of 6.1 min and a methylene unit value of 22.1 (2210 Kovats units).

Total-ion-current spectra

The total spectrum of synthetic *O*-acetyl-5-methoxytryptophol (after background subtraction and normalization) is shown in Fig. 1. Major peaks are found at *m/e* 245, 232 and 305 (*M*⁺), together with a number of very minor peaks. The proportions of the three major peaks are 100:61:21.

Fig. 1. Total spectrum of synthetic *O*-acetyl-5-methoxytryptophol (a) and of *O*-acetyl-5-methoxytryptophol in a pineal-gland extract (b)

Both spectra were obtained after g.l.c.-mass spectrometry at a retention time of 6.1 min. Major peaks occur at *m/e* 245, 232 and 305 (*M*⁺) and minor peaks at *m/e* 246, 233, 230, 202, 214, 262 and 290; some of the minor peaks are enhanced owing to contaminants that co-chromatograph. The inset structure shows the parts of the *O*-acetyl-5-methoxytryptophol molecule contributing to the three major peaks. Abbreviation used: Me₃Si, trimethylsilyl.

Single-ion-detection

All extracts and blanks were first examined at *m/e* 245 as this is the major ion fragment and the most readily detected. The night-time pineal-gland extract showed an unequivocal and fairly large peak at 6.1 min (see Fig. 2), whereas the day-time pineal-gland and all other extracts and blanks gave no peak at the correct retention time. All extracts were then examined at *m/e* 232 (Fig. 2), when the same results were obtained, thus confirming the previous data. The night-time pineal extract was then examined at *m/e* 305, as it was considered that a sufficiently large amount of *O*-acetyl-5-methoxytryptophol was present to enable the molecular ion to be seen even though it gives a

Fig. 2. *G.l.c.-mass spectrometry of extract of pineal glands collected at night*

Peaks were measured at (a) m/e 245, (b) m/e 232, and (c) m/e 305 (M^+). Good peaks are observed at the correct retention time of 6.1 min.

peak only 21% of the major ion; a peak at the correct retention was again observed. Normalized values obtained from these three peaks were well within the limit of experimental error (100:67:26).

A mass spectrum of the night-time pineal-gland extract was recorded (Fig. 1) at 6.1 min and peak proportions were found to be 100:63:23, which is in excellent agreement with the theoretical values and also indicates that there was no enhancement by background material due to interfering peaks. All the minor peaks were also present, but these invariably enhanced. Blanks and a few other tissues were also examined, but only background spectra were recorded.

Discussion

The data show conclusively that *O*-acetyl-5-methoxytryptophol occurs naturally in both male and female rat pineal glands and that there is a strong circadian rhythm involved in its synthesis, as it is present in large amounts in glands taken at night after a number of hours of darkness, whereas no detectable amount was found at midday. For obvious reasons it has not been possible to examine human glands in a like manner.

During extensive studies on circadian samples of human plasma, 5-methoxytryptophol was found in most night-time specimens and in many day-time specimens (Leone *et al.*, 1979b). As *O*-acetyl-5-methoxytryptophol is rapidly hydrolysed on incubation with human plasma with a half-life of some 2–4 min, preliminary attempts have been made to determine whether *O*-acetyl-5-methoxytryptophol occurs in plasma (by using a variety of enzyme inhibitors) and whether the concentrations of these two compounds are related.

On the basis of these data, a reasonable hypothesis on the role of *O*-acetyl-5-methoxytryptophol and its interrelationship with 5-methoxytryptophol can be advanced. It seems likely that *O*-acetyl-5-methoxytryptophol is the primary compound synthesized in the pineal gland and that any 5-methoxytryptophol found is an artefact, due either to hydrolysis by enzymes present in the blood bathing this highly vascularized organ, or to the extraction technique. If this hypothesis is correct, one might expect that the biosynthetic pathway is, by analogy with *O*-acetyl-5-hydroxymethoxytryptophol:

and a search for acetylhydroxytryptophol in the pineal gland is needed. Because of its rapid rate of hydrolysis, *O*-acetyl-5-methoxytryptophol may have a local action, e.g. in the hypothalamus. Again, because of its partial structural relationship to acetylcholine and its rate of hydrolysis by cholinesterases in plasma, it might possess neurotransmitter properties. The decrease in plasma 5-methoxytryptophol in the third part of the menstrual cycle (Hooper *et al.*, 1979a) and in hypoglycaemic stress in children (Hooper *et al.*, 1976b) suggests that its action is probably not only local (as mentioned above), but more general, or on a target organ or tissue elsewhere in the body, and this is in agreement with the known role of 5-methoxytryptophol on the sexual organs.

It now seems likely that *O*-acetyl compounds may exist more commonly than previously supposed, and that a search for such compounds could profitably be made. Clearly, *O*-acetyl-5-methoxy-

tryptophol is a compound worthy of further investigation, and the role of 5-methoxytryptophol must be reconsidered.

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