from hamsters but not those from rats [8, 17]. The present results demonstrate another difference between brown and white adipocytes from rats.

Summary. The main findings of this study were: forskolin stimulated, in a dose-dependent fashion, the accumulation of cyclic AMP, lipolysis and respiration as did isoproterenol. Clonidine (an α_2-agonist) inhibited forskolin-induced accumulation of cyclic AMP and lipolysis. The clonidine-induced inhibition of lipolysis was overcome by yohimbine (an α_2-adrenergic antagonist) in a dose-dependent manner. These results indicate that in adipocytes isolated from dorsal interscapular brown fat of rats there is an α_2-adrenergic reduction of cAMP accumulation and inhibition of lipolysis and respiration.

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The metabolism of *O*-acetyl-5-methoxy-tryptophol in the rat

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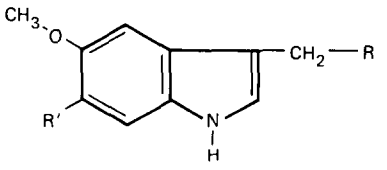
The metabolism of melatonin (aMT) (Fig. 1) is unique among pineal methoxy-indoles. It is hydroxylated at the six position [1] and subsequently excreted as the sulphate or the glucuronide conjugate. The 6-conjugates of melatonin account for over 80% of the administered dose; small quantities of unconjugated 6-hydroxy-melatonin and a kynurenine are also found. The metabolism of melatonin is rapid and relatively complex. By contrast, 5-methoxy-tryptophol (ML) and 5-methoxy-tryptamine (MT) are rapidly excreted as a single metabolite, 5-methoxy-indole-acetic acid (MIAA) with only trace quantities of unchanged ML or MT [2, 3].

In 1966 Delvigs and Taborsky [4] attempted to discover the precise structural alterations that would account for this difference of metabolism. They synthesized a novel compound, *O*-acetyl-5-methoxy-tryptophol (aML) (Fig. 1), arguing that its close structural similarity to melatonin should ensure that it would favour hydroxylation rather



	R	R'
melatonin	$CH_2NHCOCH_3$	H
O–acetyl–5–methoxy–tryptophol	CH_2OCOCH_3	H
5–methoxy–tryptophol	CH_2OH	H
6–hydroxy melatonin	$CH_2NHCOCH_3$	OH
5–methoxy–indole–acetic acid	COOH	H
5–methoxy–tryptamine	CH_2NH_2	H

Fig. 1. The structures of the principal pineal indoles and their metabolites.

than side-chain oxidation.

In fact, they discovered that the metabolism of *O*-acetyl-methoxy-tryptophol underwent both side-chain oxidation and hydroxylation. They tentatively identified this single metabolite of aML as 7-hydroxy-5-methoxyindole-3-acetic acid.

In 1980 we set up a Gas Chromatography Mass Spectrometry (GCMS) assay for 5-methoxy-tryptophol and used aML as an internal standard [5]. The assay was unsuitable because we discovered that *O*-acetyl-5-methoxy-tryptophol was rapidly being hydrolysed to 5-methoxy-tryptophol in plasma. Later we showed that the hydrolysis was caused by esterase enzymes in blood (EC 3.1.1.6) and that the half-life of *O*-acetyl-5-methoxy-tryptophol in the presence of these enzymes was less than 5 min [5]. On this basis it was difficult to understand the findings of Delvigs and Taborsky. If *O*-acetyl-5-methoxy-tryptophol is converted to 5-methoxy-trytophol by plasma enzymes it would be excreted as 5-methoxy-indole acetic acid and not as some novel metabolite.

Thus we have re-examined the work of Delvigs and Taborsky in the light of our recent findings.

Materials and methods

All solvents were of Analar Grade and obtained from B.D.H. Co. Chemicals. Thin layer chromatography (TLC) plates (Merck 5554) were purchased through B.D.H. Location reagents (Ehrlich and Gibbs), and ML and MIAA were obtained from Sigma Chemical Co., Dorset. The derivatising reagent for GCMS analysis (MSTFA) was obtained from Phase Separations, Clwyd, Wales. Wistar rats were from the Hospital Animal House, with the Sprague–Dawley rats from Charles River, U.K.

aML was synthesised according to the method described by Leone *et al.* [5].

A Varian 311A mass spectrometer interfaced to a Varian Gas Chromatograph was used to confirm the structure and purity of synthetic aML and to confirm the identity of the metabolites.

MS conditions were: filament, 1 mA; electron voltage, 75 eV, source temperature was 200° and source housing at 180°. The interface and separator were set at 250°. The GC column was a 1 m glass column packed with 10% OV1 on Chromosorb Q (100–120 mesh). The helium flow rate was 50 ml/min and the GC oven temperature was set at 240° with the injector at 260°.

Immediately prior to administration, each indole was weighed out and dissolved in 0.25 ml of ethanol. The solution was then diluted with one ml of saline which often resulted in some precipitation. A minimum volume of ethanol was then added to effect solution. The animals were injected (i.p.) at 3 p.m. and placed in a standard metabolic cage and urine collected until 10 a.m. the following day. The rats were denied food during the collection but had access to water *ad lib.* The metabolic cage consisted of a metal container with a wire mesh bottom connected to a glass separator which allowed the collection of faeces free urine.

All urines were analysed immediately after collection by TLC. Two solvent systems were used: solvent A consisted of *n*-butanol, acetic acid and water (4:1:1); solvent B was chloroform: acetic acid (19:1).

For GCMS, 2 ml aliquots of urine at pH 3 were extracted into 3 ml of ethyl acetate by vortexing for 10 sec. After settling the organic phase was transferred to a glass vial and taken to dryness under nitrogen. When dry, 250 μl of MSTFA was added and the tube heated at 60° for 10 min. Five μl aliquots were injected onto the column. Full mass spectra were obtained by scanning the magnet from mass 20 to mass 500. The spectra were recorded on u.v. sensitive paper and manually normalized.

Both location reagents (Ehrlich and Gibb's) were able to locate 1 μg of 6-hydroxy melatonin when run on a TLC plate with either solvent mixture. This test was used prior to each experiment in order to ensure the activity of the reagents.

Results

Experiment A. Four female Wistar rats were used. One was injected with ethanol:saline as a control, one with 10 mg of ML and two with 10 mg of aML.

TLC failed to differentiate between the urines from rats given ML or aML. Only one spot was visualized with solvent B when located with Ehrlich reagent at Rf 0.45, and this was in accord with the work reported by Delvigs and Taborsky [4]. Solvent A showed four spots when sprayed with Ehrlich's reagent. Two of these were present in the control, another was very weak and the major spot, which was not present in the control, had an Rf of 0.88. This major spot corresponded to MIAA. No spots were located with Gibb's Reagent.

Experiment B. Four Sprague–Dawley rats (2 male, 250–300 g) were used in this experiment. One was used as a control. The other three (2 females, 1 male) were injected with 10 mg of aML. Analysis of the urines gave identical results to Experiment A, i.e. the urines from rats given aML showed a major spot at Rf 0.45 with solvent B and a major spot at Rf 0.88 in solvent A which were not present in the control urine.

Two ml aliquots of urine from each of the animals which had been given aML were extracted and analysed by GCMS. They all showed a large peak between 3'35" to 3'40" with identical spectra to MIAA. Reference MIAA had a retention time of 3'37" and fragmented to give a spectrum with mass 232 at the base peak. The total spectrum was: 232(100%), 306(4%), 334(7%) and the molecular ion (as the TMS derivative) at 349(17%).

Experiment C. Seven female Wistar rats (200 g) were used in this experiment, two were injected with 10 mg of ML, two with 10 mg of aML and two with 10 mg of MIAA and one as control. No significant difference could be shown using TLC between the urines from the animals given aML, ML, or MIAA apart from a small quantity of unchanged ML(<2%) seen in a urine from a rat loaded with ML. GCMS analysis again showed the major extractable compound to be MIAA. Comparison of the peak heights of the MIAA peak from extracts of the urines from rats given ML and aML established that equivalent quantities were produced. From this it was clear that MIAA is the only metabolite of note from aML and when aML or ML are administered in equal doses, the same relative quantity of MIAA is produced as a metabolite.

Finally urines from rats given aML and MIAA were extracted at various pH values. Two ml aliquots were extracted at pH 3, pH 5.5 and also at pH 6.5, the pH of

unchanged urine. They were run in solvent B and the spots located with Ehrlich's reagent. Maximum recovery was achieved at pH 3 for both MIAA and aML loaded urines. It was estimated that <10% was recovered from neutral urine, whereas >80% was recovered at pH 3.

Discussion

Melatonin is unique among pineal methoxy-indoles in having a metabolic pathway consisting primarily of aromatic hydroxylation. By contrast ML and MT are oxidised to MIAA. This study has shown unequivocally that aML is also metabolised to MIAA. The evidence can be summarised as follows:

(a) Whether rats are given aML, ML or MIAA they produce the same major urinary product as analysed by TLC and GCMS.
(b) The single urinary product has the same TLC Rf, the same GC retention time and the same mass spectrum as MIAA.
(c) The major urinary product is not altered by changing the sex or species of the rat.

It may therefore be surprising that Delvigs and Taborsky found that aML was metabolised differently from ML. In their experiment, they located a spot using Gibb's reagent and this was the principal evidence they adduced to support the idea that the metabolite contained a phenol group. One possible reason for their finding is that they used synthetic aML which was in the form of an oil and consequently probably contained an impurity and it is possible that such an impurity gave rise to the reaction with the Gibb's reagent.

Another possibility is that injection of aML as the picrate salt could in some way block the blood esterases and prevent the conversion of aML to ML in the circulation. In this case aML could indeed be metabolised differently from ML. However such a possibility is simply a conjecture and would need to be proved.

In summary, when aML was administered to rats the urinary metabolite was found to be MIAA and was identified using TLC and GCMS. This disproves the hypothesis that aML undergoes hydroxylation as is the case for melatonin.

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The effects of metal ions on the binding of a new α_2-adrenoceptor antagonist radioligand (^{3}H)-RX 781094 in rat cerebral cortex

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At least two types of α-adrenoceptors (α_1 and α_2) can be demonstrated in tissues [1–4]. In the brain, radioligand binding techniques have made it possible to characterise α-receptor systems; (^{3}H)-prazosin has been used to identify α_1-adrenoceptors [5, 6], whilst α_2-adrenoceptors have been studied with (^{3}H)-clonidine [7, 8], (^{3}H)-*p*-aminoclonidine [9], (^{3}H)-yohimbine [6, 10], (^{3}H)-guanfacine [11] and [^{3}H]-rauwolscine [12].

Recently, properties of a novel α_2-adrenoceptor antagonist, RX 781094 [2-(2-(1,4-benzodioxanyl))-2-imidazoline HCl], have been described; this compound has been shown to be potent and more α_2-selective than yohimbine [13] and to have activity in the CNS [14–16]. (^{3}H)-RX 781094 has been prepared and has been shown to label α_2-adrenoceptors in rat brain [17, 18]. Tris–HCl buffers have been used extensively in binding studies, but in some, the addition of metal ions changed the binding characteristics of the radioligand [10, 11, 19, 20]. We report here the effects of mono- and divalent metal ions added to Tris–HCl buffer on the binding of (^{3}H)-RX 781094 to membranes of rat cerebral cortex; this has been compared with the binding obtained using a buffer comprising physiological concentrations of metal ions.

Materials and methods

(^{3}H)-RX 781094 HCl was prepared by catalytic bromine–tritium exchange (Amersham International Ltd.) and purified by preparative TLC (>99%). The specific activity was 30 Ci/mmol.

The physiological salt solution contained (mM): NaCl, 118; KCl, 4.8; $CaCl_2$, 1.3; KH_2PO_4, 1.2; $MgSO_4$, 1.2; $NaHCO_3$, 25; equilibrated at 25° with 95% O_2/5% CO_2 before use; final pH 7.4.

Brain tissue was obtained from male Sprague–Dawley rats, 200–250 g (Bantin and Kingman Ltd., U.K.) killed by cervical dislocation. Rat cerebral cortex was dissected free from striatal tissue but included hippocampus. Brain tissue was homogenized in 10 vol. (w/v) of buffered sucrose (0.3 M in 0.05 M Tris–HCl pH 7.8) using a Potter-type Teflon–glass homogenizer. A crude synaptosomal P_2-fraction was prepared according to the procedure of Whittaker [21]. The P_2-pellet was washed with 0.05 M Tris–HCl pH 7.8, and the membranes centrifuged at 10,000 *g* for 15 min. Membranes were resuspended in the physiological salt solution or in 0.05 M Tris–HCl pH 7.8 or in 0.05 M Tris–HCl pH 7.8 with added metal salt to give a final protein concentration of approx 1 mg/ml. Protein assays were per-