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 $\alpha_2$-adrenoceptor antagonist radioligand ($^3$H)-RX 781094 in rat cerebral cortex

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At least two types of $\alpha$-adrenoceptors ($\alpha_1$ and $\alpha_2$) can be demonstrated in tissues [1–4]. In the brain, radioligand binding techniques have made it possible to characterise $\alpha$-receptor systems; ($^3$H)-prazosin has been used to identify $\alpha_1$-adrenoceptors [5, 6], whilst $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_2$-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-

formed as described by Lowry *et al.* [22].

Binding assays were carried out in triplicate. The incubation mixture contained synaptosomal membranes (970 μl), water or saturating ligand or competing ligand (10 μl) and ($^3$H)-ligand (20 μl). The mixture was incubated at 25° for 15 min. The membranes were then quickly filtered through Whatman GFB filters, which were rapidly washed twice with 4 ml of 0.05 M Tris–HCl buffer pH 7.8. The filters were then placed in vials containing 3.5 ml of NE 260, scintillation fluid (Nuclear Enterprises) and counted for radioactivity. Specific binding was defined with L-adrenaline bitartrate (300 μM) or phentolamine mesylate (1 μM) or yohimbine HCl (2 μM) all of which gave comparable results; no displacement of binding was observed with prazosin (100 nM).

The Hanes semi-reciprocal plot (free vs free/bound) was used to estimate the affinity constant ($K_D$) and the concentration of binding sites ($B_{max}$). This plot gives $B_{max}$ from the reciprocal of the slope and $K_D$ from the $X$-intercept [23].

*Results and discussion*

These studies have investigated the effect of changing the incubation medium on the binding of ($^3$H)-RX 781094 to rat cerebral membranes. All membranes were prepared in the same way using an isotonic buffer to give a $P_2$-fraction followed by a hypotonic step to lyse the membranes. A common incubation time of 15 min was adopted since this was convenient; preliminary studies showed that at 25° in Tris–HCl, the association and dissociation of ($^3$H)-RX 781094 was rapid ($t_{1/2} < 1$ min). All filters were washed with 2 × 4 ml washes of Tris–HCl pH 7.8; up to 3 × 4 ml washes could be carried out without reducing the amount of specific binding of ($^3$H)-RX 781094. The effect of changing the pH of Tris–HCl over the range 7.1–8.0 showed that similar amounts of specific binding were obtained at each pH with maximal binding occuring at pH 7.8.

*The effects of metal ions on the amount of saturable binding of* ($^3$H)-*RX* 781094. In Tris–HCl pH 7.8 the specific binding of ($^3$H)-RX 781094 (1 nM) was between 59 and 71% of total binding (mean 62 ± 2%). Sodium chloride (1–10 mM final concentration) added to the Tris–HCl incubation medium produced increases in the amount of specific binding of ($^3$H)-RX 781094 (Fig. 1). With higher concentrations (50–100 mM) the amount of specific binding was similar to that produced using Tris–HCl alone. Similar increases in the specific binding of ($^3$H)-RX 781094 were observed whether sodium ions were added as sodium chloride or sodium glycerophosphate (Fig. 1). High concentrations of potassium ions added to the Tris–HCl incubation medium produced marked (40–50%) reductions in the amount of specific binding; the reduction at the physiological potassium ion concentration (6 mM) was however only small (17%, Fig. 1) and not significantly different from control. Calcium and magnesium ions when added to the Tris–HCl incubation medium both produced concentration dependent reductions in the amount of specific binding of ($^3$H)-RX 781094 (Fig. 1); 1.3 mM calcium and 1.2 mM magnesium reduced the level of specific binding by 43% and 47% respectively.

These results, obtained using the $\alpha_2$-antagonist ligand, ($^3$H)-RX 781094 are opposite to those seen by others using $\alpha_2$-agonist radioligands [11, 19, 20]. Those workers showed that sodium ions decreased the amount of specific binding, whereas divalent metal ions such as magnesium and calcium increased specific binding.

*The effects of metal ions on the values of* $B_{max}$ *and* $K_D$ *obtained with* ($^3$H)-*RX* 781094. In 50 mM Tris–HCl, the specific binding of ($^3$H)-RX 781094 (0.1–10 nM) to the

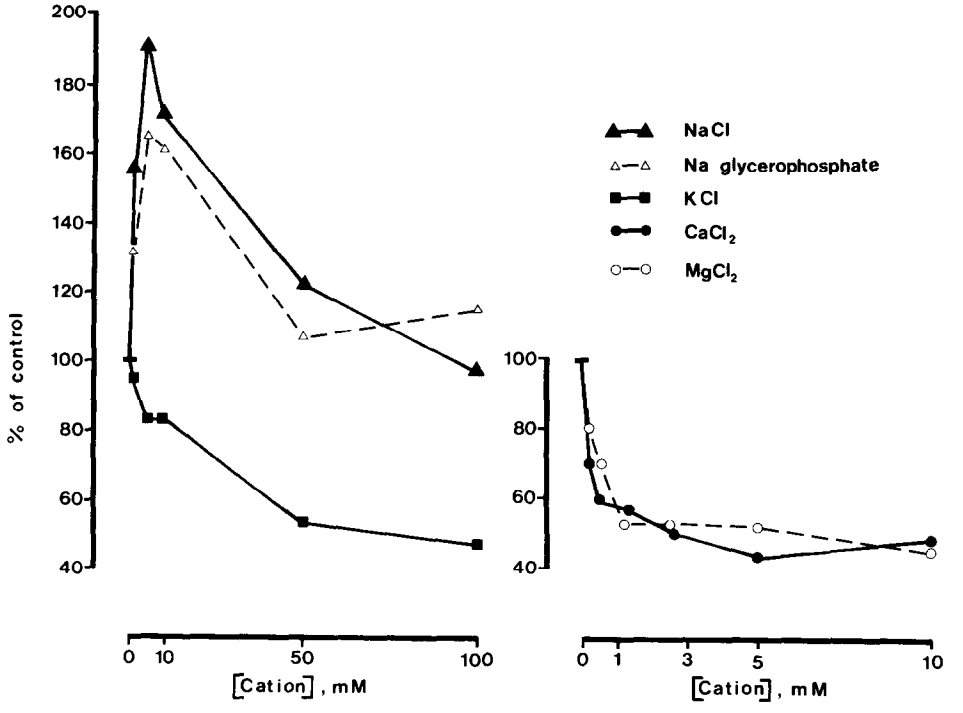


Fig. 1. The effects of cations on the amount of saturable binding of ($^3$H)-RX 781094 to rat cortex $P_2$-fraction membranes, expressed as a percentage of the amount of binding which occurred in 50 mM Tris–HCl pH 7.8 in the absence of added cations.

cerebral cortex $P_2$-fraction appeared to be to more than one site, as indicated from the Hanes plot which was curvilinear (Fig. 2). The total concentration of sites labelled was about 180 fmole/mg protein and the data suggests that these were divided between high affinity (2 nM) and lower affinity sites (Table 1). With the addition of 5 mM NaCl or 143 mM NaCl or 6 mM KCl to the Tris–HCl incubation medium, a single population of high affinity sites was indicated (Table 1), whereas the addition of either 1.3 mM $CaCl_2$ or 1.2 mM $MgCl_2$ to Tris–HCl produced a marked decrease in the concentration of sites labelled by ($^3$H)-RX 781094 (Table 1), as shown by the increased slope of the Hanes plot (Fig. 2). In a similar study, Rouot *et al.* [10] have shown that 1 mM concentrations of calcium, magnesium or manganese ions reduce the concentration of ($^3$H)-yohimbine binding sites in rat brain membranes.

*Binding characteristics of* ($^3$H)*-RX* 781094 *in the physiological salt solution.* In the physiological salt solution the specific binding of ($^3$H)-RX 781094 (0.1–35 nM) to the rat cortex $P_2$-fraction was a saturable process (Fig. 2). A single population of sites $K_D = 4.6 \pm 0.6$ nM (Table 1) was indicated from the data, transformed using the Hanes semi-reciprocal plot, $B_{max} = 153 \pm 15$ fmol/mg protein. Langer *et al.* [24] found similar binding characteristics for ($^3$H)-RX 781094 in 50 mM sodium potassium phosphate buffer ($B_{max} = 189 \pm 13$ fmol/mg protein, $K_D = 3.9 \pm 0.4$ nM. The range of concentrations of $\alpha_2$-binding sites in rat cortex reported in the literature [7–11] is wide (90–242 fmol/mg protein) since workers have used different incubation media and have purified the membrane preparations to different extents. The concentration of $\alpha_2$-binding sites found in the present study was within this range of values.

There is evidence, both from studies with platelets [25] and with cerebral membranes [26, 27], that the $\alpha_2$-adrenoceptor can exist in at least two states and that the higher affinity state probably exists as a complex of receptor and adenylate cyclase regulatory protein. A further "super-high affinity" state of the $\alpha_2$-adrenoceptor has been reported to be induced by magnesium ions; in competition binding experiments with ($^3$H)-clonidine, GTP increased and magnesium reduced the affinities of $\alpha_2$-antagonists for this state of the $\alpha_2$-receptor [28]. In the present study, the reduction in the concentration of ($^3$H)-RX 781094 binding sites caused by divalent metal ions might represent a partial shift of the $\alpha_2$-binding sites to the "super-high affinity" state; RX 781094 might have a very low affinity for this site and would thus label fewer sites. The physiological relevance of such a site is questionable, however, since a reduction in $\alpha_2$-binding sites is not seen when the divalent metal ions are mixed with monovalent ions to form the physiological incubation medium.

Since formation of ligand–receptor complexes appear to be influenced by the mix of metal ions and nucleotides in the incubation medium, the choice of the medium for

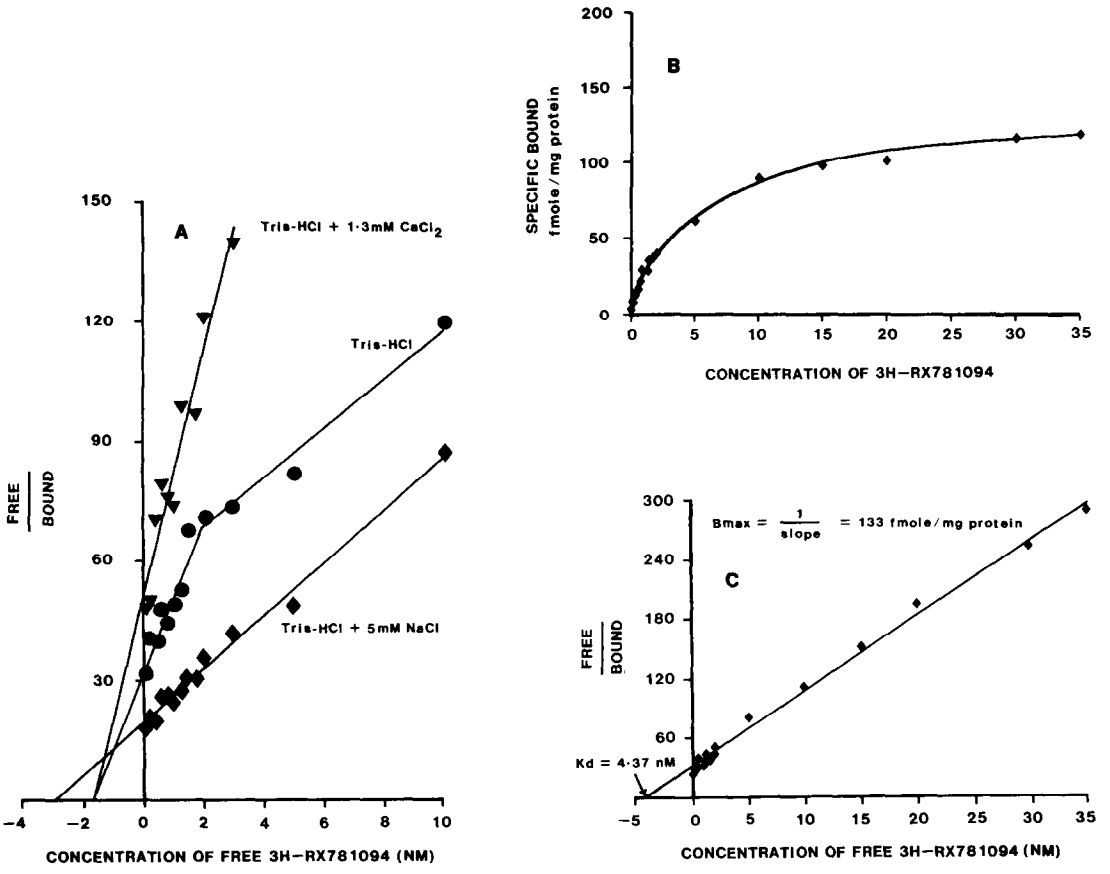


Fig. 2. (a) Representative Hanes plots of binding data in various incubation media: a comparison of 50 mM Tris–HCl (●——●), with Tris–HCl + 5 mM NaCl (◆——◆) and with Tris–HCl + 1.3 mM $CaCl_2$ (▼——▼). Intersection on the abscissa gives an estimate of the $K_D$ value. $B_{max}$ is obtained from 1/slope. Hanes plots of Tris–HCl + 1.2 mM $MgCl_2$ data were very similar to those of Tris–HCl + 1.3 mM $CaCl_2$. (b) Specific binding of ($^3$H)-RX 781094 (0.1–35 nM) in the physiological salt solution (data from one experiment). (c) The Hanes secondary plot of the data represented in (b).

Table 1. Values of $B_{max}$ and $K_D$ obtained from the Hanes linearisation of specific binding of ($^3$H)-RX 781094 to a rat cortex $P_2$ fraction, carried out in a number of incubation media. Values are the mean ± S.E.M.

| Incubation medium | Concentration range (nM) | $N$ | $B_{max}$ (fmol/mg protein) | $K_D$ (nM) |
|---|---|---|---|---|
| Tris–HCl | 0.1–10 | 5 | 181 ± 31 | 12.3 ± 1.5 |
| Tris–HCl | 0.1–2 | 5 | 47 ± 10 | 2.0 ± 0.6 |
| Tris–HCl + 5 mM NaCl | 0.1–10 | 3 | 131 ± 6 | 3.4 ± 0.3 |
| Tris–HCl + 143 mM NaCl | 0.1–10 | 3 | 179 ± 17 | 4.6 ± 0.2 |
| Tris–HCl + 6 mM KCl | 0.1–10 | 3 | 185 ± 25 | 4.9 ± 0.9 |
| Tris–HCl + 1.2 mM $MgCl_2$ | 0.1–10 | 3 | 38 ± 4 | 3.0 ± 0.2 |
| Tris–HCl + 1.3 mM $CaCl_2$ | 0.1–10 | 3 | 57 ± 3 | 4.4 ± 0.2 |
| Physiological salt solution | 0.1–35 | 5 | 153 ± 15 | 4.6 ± 0.6 |

competition binding studies becomes important. For instance underestimates of affinities of agonists for sites labelled by ($^3$H)-antagonists could occur, and vice versa for sites labelled by ($^3$H)-agonists, depending on the choice of incubation medium. The question of whether binding should be optimized by manipulating the ionic environment, or whether physiologically relevant incubation media should be used, remains open. Our confidence in using a physiological incubation medium routinely has, however, increased with the observation that there was a good correlation between the affinities of $\alpha_2$-antagonists obtained from ($^3$H)-RX 781094 binding studies in this medium and those determined using the isolated rat vas deferens preparation [18].

In summary, the binding of the novel $\alpha_2$-adrenoceptor antagonist ($^3$H)-RX 781094 to rat cerebral cortex membranes has been studied using a number of incubation media. The inclusion of magnesium or calcium ions in the Tris–HCl medium markedly reduced the apparent concentration of binding sites. When binding was carried out in an incubation medium comprising physiological concentrations of both divalent and monovalent metal ions, ($^3$H)-RX 781094 bound to a single population of $\alpha_2$-binding sites, $K_D = 4.6 \pm 0.6$ nM, $B_{max} = 153 \pm 15$ fmol/mg protein.

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