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Ascorbic acid and membrane ageing: critical determinants of the in-vitro binding of [^{3}H]ADTN to rat striatal tissue

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Tritiated (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene ([^{3}H]ADTN), binding (0·125–4·0 nM) to rat striatal membranes was investigated using Tris-HCl buffer containing Na_2EDTA, nialamide and varying concentrations of ascorbic acid. In the absence of ascorbic acid [^{3}H]ADTN exhibited a high affinity (K_D 1·26 nM) saturable binding (B_{max} 138 fmol mg^{-1} protein) (Scatchard analysis). This was not modified by 10^{-6} or 10^{-5} M ascorbic acid used immediately or 60 min after preparation. However, 10^{-4} M ascorbic acid added (*within*) 15 min after its preparation reduced the number of bindings sites and added 60 min after it also reduced affinity. Ascorbic acid 5.7 mM reduced affinity whether used *within* 15 min or 60 min of its preparation. When ascorbic acid 10^{-4} M was used *within* 15 min of preparation, and membranes were used immediately, the binding of 2 nM [^{3}H]ADTN was specifically displaceable by nM or sub-nM concentrations of dopamine agonists and antagonists. However, when membranes were used 1–2 h after their preparation there was an increasing loss of the high affinity binding sites displaceable by sub-nM concentrations of (±)-ADTN. Thus, [^{3}H]ADTN can be shown to exhibit high affinity stereoselective binding to rat striatal membranes when these are freshly prepared and the assay is performed using Tris-HCl buffer containing nialamide, Na_2EDTA and 10^{-4} M ascorbic acid prepared within 15 min of use. The characteristics of this binding can be markedly modified if the concentration of ascorbic acid is increased, if its preparation time is extended, or if the membranes are allowed to 'age'.

Numerous investigators using tritiated neuroleptic agents as ligands to identify putative dopamine receptors in the rodent striatum have consistently demonstrated saturable high affinity binding displaceable by appropriately low concentrations of other neuroleptic agents. However, the use of tritiated dopamine agonists to label putative dopamine receptors in rat striatal tissue has proved unexpectedly difficult. Whilst some authors report that high concentrations of non-labelled dopamine agonists and antagonists are required to inhibit the binding of dopamine agonist ligands to rat striatal tissue (Creese & Snyder 1978; Creese et al 1979; Davis et al 1980; Seeman et al 1979) others show potent displacements (Leysen 1980; Fujita et al 1980; Sokoloff et al 1980; List et al 1982). Whilst non-specific binding may have complicated a number of studies (Leysen & Gommeren 1981), the most controversial finding is that ascorbic acid, a constituent of almost all binding assay buffers, can itself modify dopamine agonist binding (Kayaalp & Neff 1980; Leff et al 1981; Zahniser et al 1981; Bacopoulos 1982; Heikkila et al 1982). Most studies show ascorbic acid to reduce dopamine agonist/antagonist binding, but the interpretation of this effect has differed widely with, for example, Kayaalp et al (1981) considering the presence of ascorbate to effectively preclude the use of [^{3}H]dopamine in binding assays and Leff et al (1981) suggesting that ascorbic acid is actually required to demonstrate a specific binding to dopamine receptors. Hence, present studies were designed to analyse the effects of including ascorbic acid in the assay medium for the binding of [^{3}H]ADTN to rat striatal membranes.

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MATERIALS AND METHODS

Female, Sprague-Dawley (CD) rats, 250 ± 25 g, were stunned, the brains removed and the striata (80 mg per rat) dissected on ice. The striata were homogenized (Polytron setting '5', 10 s) in 50 vols ice cold Tris-HCl buffer (50 mM, pH 7.4 at 25 °C) and centrifuged (50 000 *g* 3 min), resuspended in the same buffer (Polytron setting '5', 5 s) and recentrifuged. The pellet was resuspended (Polytron setting '5', 5 s) in 50 vols Tris-HCl buffer (50 mM, pH 7·4 at 37 °C) and incubated with agitation for 12 min at 37 °C. The suspension was cooled on ice and centrifuged/resuspended in 50 vols Tris-HCl buffer

(50 mM, pH 7.4 at 25 °C) before final resuspension (Polytron setting '5', 10 s) in a Tris-HCl buffer (50 mM, pH 7.4 at 25 °C) containing nialamide (10 μM) and Na_2EDTA (5 mM). The entire procedure of membrane preparation was accomplished in 70–75 min.

The membrane suspension (10 mg ml^{-1} wet weight tissue) was gently agitated (Scienco Magnetic Stirrer) on ice and, unless stated otherwise, was used immediately. Assay tubes, on ice, received (a) 0·5 ml membrane suspension (equivalent to approximately 250 μg protein determined by the method of Lowry et al 1951, with correction for the fact that Tris-HCl ascorbic acid buffer solution can exaggerate the 'protein' determinations by 25–30%, see also Sokoloff et al 1980), and (b) ascorbic acid/buffer 0.2 ml, (c) displacing drug/buffer 0.2 ml and (d) ligand 0.2 ml to a final volume of 1·1 ml.

The experimental design for I, II and IV (see Results) using relatively small numbers of tubes allowed completion of ascorbic acid additions (immediately before ligand/displacing drug) within 2 min, whilst the addition of ascorbic acid to the large numbers of tubes used in III, V and VI for Scatchard analyses and the construction of displacement curves was completed in 5–15 min.

Final incubation was for 15 min at 25 °C. Bound and free ligand were then separated by rapid filtration through Whatman GF/B filters, washed with 2 × 5 ml ice-cold Tris-HCL buffer. The filters were placed in 5 ml Instagel (Packard Instruments) and vigorously shaken for 20 min (IKA-VIBRAX-VXR, Janke and Kunkel). After temperature and light adaptation the samples were counted by liquid scintillation spectroscopy (Packard-Tricarb, model 460C) at an efficiency of approximately 45%. Specific binding of [^{3}H]ADTN was defined as the total binding minus the binding occurring in the presence of dopamine agonists and antagonists.

Tritiated (±)-2-amino-6,7-dihydroxy-1,2,3,4-tetrahydronaphthalene. HCl ([^{3}H]ADTN) was obtained from New England Nuclear, 36·3, 35·2 and 28·3 Ci $mmol^{-1}$ (whilst a chromatographic analysis indicated the radio-chemical purity of [^{3}H]ADTN to be greater than 97%, all samples were routinely tested for an ability to produce saturable binding displaceable by low nM concentrations of dopamine). Ligands were used within 2 months of receipt (storage at −20 °C). The ligands were prepared in N_2 bubbled ice cold buffer immediately before addition to the incubation tubes. Similar precautions were taken for the preparation of cold (±)-ADTN (Lundbeck), dopamine HCl (Koch Light), apomorphine HCl (Macfarlan Smith), (−)*N*-n-propylnorapomorphine HCl, (−)NPA, (Research Biochemicals Inc.), noradrenaline hydrogen tartrate (Sigma), isoapomorphine HCl (Dr R. M. Pinder), catechol (BDH), fluphenazine HCl (Squibb), *cis*(Z)- and *trans*(E)-flupenthixol 2HCl (Lundbeck), metoclopramide monohydrochloride (Beechams), (±)-propranolol HCl (ICI), piperoxan HCl (Roche) and yohimbine HCl (Sigma). Domperidone and haloperidol (Janssen), (+)- and (−)-sulpiride (SESIF, France) and (+)- and (−)-butaclamol (Ayerst) were similarly prepared with a further addition of a minimum quantity of *NN*-dimethylformamide. Nialimide (Sigma), Na_2EDTA (Sigma) and Tris-HCl (Sigma) were prepared in glass distilled water.

RESULTS

I. *Preliminary determination of the effect of ascorbic acid on the binding of 2 nM [^{3}H]ADTN*

The binding of 2 nM [^{3}H]ADTN was determined in the presence of 10^{-6}, 10^{-5}, 10^{-4}, 10^{-3} and $5·7 \times 10^{-3}$ M ascorbic acid added to the assay tubes 2 or 60 min after preparation. The 'specific' binding was assessed using dopamine and *cis*-flupenthixol (10^{-5} M) which had identical displacement efficacies (only data obtained with dopamine are shown in Fig. 1). The total binding of [^{3}H]ADTN was not reduced by 10^{-6} or 10^{-5} M ascorbic acid (prepared 2 or 60 min before the addition of ligand), specific binding accounting for approximately 50–60% of total binding. Similar data were obtained for 10^{-4} M ascorbic acid added within 2 min of its preparation, although added at 60 min after its preparation, this concentration reduced total binding by approximately 35%. The use of ascorbic acid 10^{-3} M prepared for 2 or 60 min caused modest reductions ($P < 0·05$) in total binding. The absolute levels of 'specific' binding were always reduced by the inclusion of ascorbic acid at concentrations higher than 10^{-3} M, notwithstanding that at 10^{-4}, 10^{-3} and $5·7 \times 10^{-3}$ M ascorbic acid (60 min preparation) the percent of specific binding actually increased slightly to 65–70% as compared with a specific binding of 50–60% recorded using the lower concentrations of ascorbic acid.

II. *The effect of ageing of ascorbic acid-buffer solution (10^{-4} M) on its ability to modify the binding of 2 nM [^{3}H]ADTN*

Binding of 2 nM [^{3}H]ADTN to rat striatal membranes was assessed in the presence of ascorbic acid prepared 0–120 min previously, and in the absence of any ascorbic acid. 'Specific' binding was assessed using 10^{-5} M dopamine as displacing agent. The total

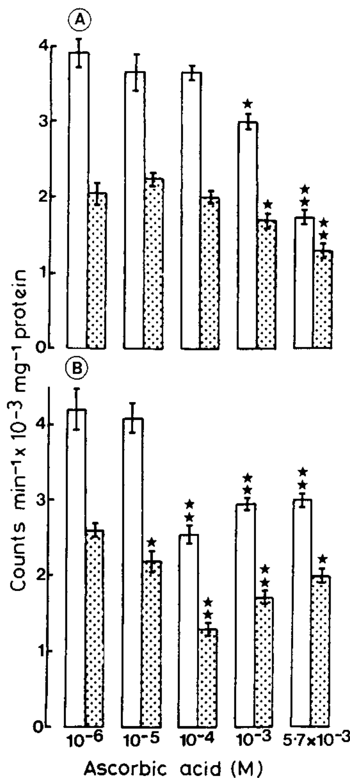

FIG. 1. Displacement of [³H]ADTN binding to rat striatal membranes (2nM, open columns, total binding) by dopamine (10^{-5} M, stippled columns, 'specific' binding) in the presence of 10^{-6}–$5{\cdot}7 \times 10^{-3}$ M ascorbic acid prepared (A) immediately or (B) 60 min before addition to the incubation tubes (i.e. before addition of ligand). n = 4–8. s.e.m.s given. Significant reductions in total and specific binding by ascorbic acid are determined by comparison with the total and specific binding respectively observed in the presence of 10^{-6} M ascorbic acid and are indicated as $*P < 0{\cdot}01$, $**P < 0{\cdot}001$ (Student's t-test).

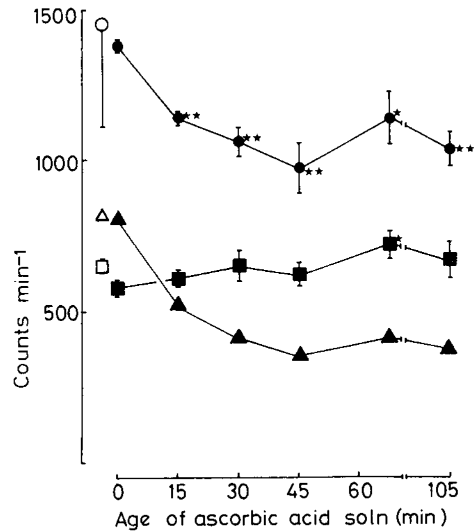

FIG. 2. The effect of ageing of ascorbic acid solution on the binding of [³H]ADTN (2 nM) to rat striatal membranes. The binding of [³H]ADTN in the absence (open symbols) or presence of 10^{-4} M ascorbic acid (closed symbols) is expressed as counts min^{-1} (counts per filter-filter blank) for total binding (○, ●——●), binding obtained in the presence of 10^{-5} M dopamine (□, ■——■) and 'specific' binding (△, ▲——▲) (total binding-binding in presence of dopamine). n = 4–8. s.e.m.s given. The significance of age of ascorbic acid solution (15–105 min) on each measure of binding is determined by comparison with data obtained using ascorbic acid prepared immediately before use (zero time), and is indicated as $*P < 0{\cdot}01$, $**P < 0{\cdot}001$ (Student's t-test).

binding of 2 nM [³H]ADTN in the absence of ascorbic acid was *approximately* 1400 counts min^{-1}, the standard error on the mean value being large, ca 20%. The use of ascorbic acid prepared at time 'zero' did not significantly change total binding but dramatically reduced the standard error of the response to 1·5–6% (this 'regularization' in binding was seen in all subsequent experiments). The use of an ascorbic acid solution that had been prepared for only 15 min caused a modest, but highly significant ($P < 0{\cdot}001$), reduction (approximately 21%) in total binding, and maximum reductions were recorded using ascorbic acid/buffer solutions prepared for 30+ min. A reduction in the specific binding was associated with the decline in total binding (Fig. 2).

III. *Modification by ascorbic acid of the saturability of [³H]ADTN binding*

Concentrations of ascorbic acid of 10^{-4} M (selected as causing a regularization in binding, no reduction in binding and yet having a reasonable antioxidant capability when freshly prepared) and 5·7 mM (the concentration routinely employed in most studies) were selected for detailed assessment of the effects of ascorbic acid, prepared for 15 or 60 min, on [³H]ADTN binding to rat striatal membranes, comparisons being made with the binding occurring in the absence of ascorbic acid. With dopamine (10^{-5} M) as the displacing agent, a saturable binding of [³H]ADTN (0·125–4 nM) was shown to occur in the absence of ascorbic acid, K_D 1·26 nM, B_{max} 138 fmol mg^{-1} protein (Fig. 3B). The most striking feature of this binding was the large standard errors on the means values (ca 15%), and the spread of values used to construct the Scatchard plot (regression coefficient 0·81). This must be contrasted with the saturability of the binding shown to occur in the presence of a treatment with fresh ascorbic acid solution (10^{-4} M), standard errors being less than

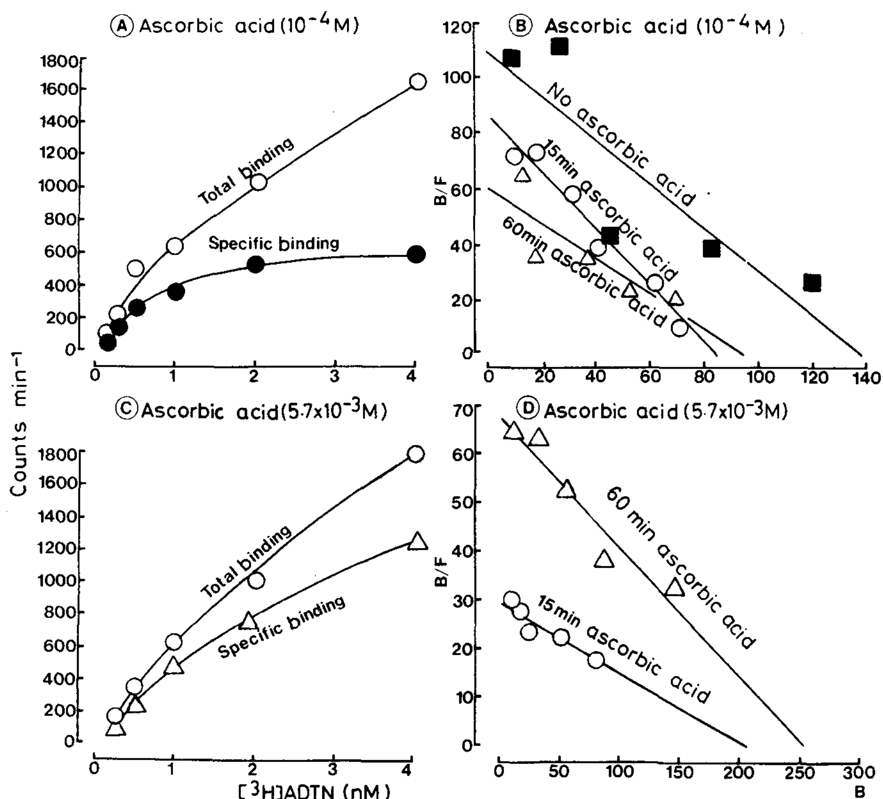

FIG. 3. Binding of [^{3}H]ADTN to rat striatal membranes (0·125–4·0 nM) in the presence of 10^{-4} M ascorbic acid (A, B) and 5·7 × 10^{-3} M ascorbic acid (C, D). A ○——○ total binding and ●——● specific binding obtained in the presence of 10^{-5} M dopamine, the ascorbic acid (10^{-4} M) being prepared 15 min before use. B Scatchard analyses of the data obtained in A. ○——○, or when ascorbic acid had been prepared for 60 min △——△, or omitted completely ■——■. C ○——○ total binding and △——△ specific binding obtained in the presence of 10^{-5} M dopamine, the ascorbic acid (5·7 × 10^{-3} M) being prepared 60 min before use. D Scatchard analyses of the data obtained in C △——△, or when ascorbic acid had been prepared for 15 min ○——○. Data in A and C are given as counts min^{-1}, derived as counts per filter-filter blank; in B and D, B (bound [^{3}H]ADTN) are given in fmol mg^{-1} protein and B/F (F being 'free' [^{3}H]ADTN) are given in fmol mg^{-1} protein nM^{-1}. n = 4–8. s.e.m.s of values determined in the presence of ascorbic acid were in the range 0.1–10%, whilst those obtained in the absence of ascorbic acid range from 8–25%.

10%, and the closeness of fit of the Scatchard plot (regression coefficient 0·98) (Fig. 3B). Whilst the K_D value of 0·98 nM was similar to that recorded in the absence of ascorbic acid, the number of specific binding sites was less, 85 fmol mg^{-1} protein. With the buffer solution of ascorbic acid prepared for 60 min standard errors on the mean values were small (<12%), the saturable binding (regression coefficient 0·85) showing a reduced affinity of [^{3}H]ADTN for the receptor (K_D 1·58 nM) compared with results using the fresh solution, but with a comparable B_{max} value of 95 fmol mg^{-1} protein.

With the higher concentration of 5·7 mM ascorbic acid a contrasting profile was observed. Firstly, the binding normally occurring (in the absence of ascorbic acid or in its presence at 10^{-4} M) at 0.125 nM [^{3}H]ADTN was abolished by the use of 5·7 mM ascorbic acid prepared for 15 or 60 min. However, the 60 min preparation only modestly reduced the total binding occurring at 2 and 4 nM [^{3}H]ADTN, and increased the proportion of [^{3}H]ADTN specifically bound (e.g. 68% at 2 nM [^{3}H]ADTN, and 70%, at 4 nM which suggests that binding was not saturated at this concentration) (Fig. 3C). The Scatchard projection indicated an approximate 3-fold increase in B_{max} (254 fmol mg^{-1} protein) relative to values obtained using 10^{-4} M ascorbic acid prepared for 60 min, although these 'specific' binding sites showed a marked decrease in affinity (K_D 3·73 nM) for [^{3}H]ADTN. With 5·7 mM ascorbic acid prepared for 15 min total binding was reduced by approximately 50% at *all* concentrations of [^{3}H]ADTN

although, as with the effect of the 60 min preparation, the percentage of specific binding was high, 67–73% at the higher concentrations of [^{3}H]ADTN. Again, a Scatchard projection indicated a saturable binding but with a B_{max} of 207 fmol mg^{-1} protein and a markedly reduced affinity, 7·2 nM (Fig. 3D).

In a more restricted series of experiments using *cis*-flupenthixol (10^{-5} M) as displacing agent, and 10^{-4} M ascorbic acid, a saturable binding of [^{3}H]ADTN was shown (with ascorbic acid prepared for 15 min—B_{max} 86 fmol mg^{-1} protein, K_D 1·17 nM, regression coefficient 0·88; for the 60 min preparation—B_{max} 85 fmol mg^{-1} protein, K_D 1·4 nM, regression coefficient 0·82; results not shown). The modification of K_D and B_{max} values for [^{3}H]ADTN binding using the various preparations of ascorbic acid are summarized in Table 1.

Table 1. Modification of K_D (nM) and B_{max} (fmol mg^{-1} protein) values for [^{3}H]ADTN binding (0·125–4 nM) using various treatments with ascorbic acid.

Ascorbic acid (preparation)	Displacing Agent	K_D	B_{max}
None	Dopamine	1·26	138
10^{-4} M (15 min)	Dopamine	0·98	85
10^{-4} M (15 min)	*cis*-Flupenthixol	1·17	86
10^{-4} M (60 min)	Dopamine	1·58	95
10^{-4} M (60 min)	*cis*-Flupenthixol	1·40	85
$5{\cdot}7 \times 10^{-3}$ M (15 min)	Dopamine	7·20	207
$5{\cdot}7 \times 10^{-3}$ M (60 min)	Dopamine	3·73	254

IV. *The potency of dopamine in displacing the binding of 2 nM [^{3}H]ADTN: the effect of ascorbic acid*

The binding of [^{3}H]ADTN and its displacement by $3 \times 10^{-11}-10^{-6}$ M dopamine was assessed in the presence of 10^{-4} M ascorbic acid prepared for 5 or 60 min. The displacement caused by dopamine in the presence of ascorbic acid prepared for 5 min was apparent at subnanomolar concentrations, 10^{-9} M dopamine causing displacement of 30% of the total binding, 10^{-6} M displacing 65% of the total binding. In the presence of ascorbic acid prepared for 60 min the effectiveness of dopamine in displacing [^{3}H]ADTN was reduced, at 10^{-9} M dopamine was ineffective and at higher concentrations it was 5–50 times less potent, the displacement curve being consistently shifted to the right (results not shown).

V. *Displacement of the binding of 2 nM [^{3}H]ADTN by dopamine agonists and antagonists*

Incubation of 2 nM [^{3}H]ADTN in the presence of $8 \times 10^{-12}-10^{-6}$ M concentrations of dopamine agonists ((±)-ADTN, (−)-NPA, dopamine, apomorphine) and related compounds (noradrenaline, isoapomorphine, catechol) was always carried out in the presence of 10^{-4} M ascorbic acid used within 15 min of preparation. The displacement curves were constructed without attempt to present lines of best fit, and only data obtained using (−)-NPA and dopamine is shown (Fig. 4; see also Fig. 5 for displacement caused by (±)-ADTN). It is emphasized that the standard errors on the mean values were less than 3%, and so the displacements recorded at subnanomolar concentrations, even those of only 10–15%, were invariably significant. Such displacements appear to plateau, but this would only be reliably demonstrated by the use of extensive concentration ranges of displacing drugs. Two points may be made with regard to the displacements caused by the dopamine agonists, (i) a significant proportion (30–40%) of total binding was displaced by $10^{-11}-10^{-9}$ M ADTN, (−)-NPA and dopamine, and (ii) displacement curves for higher concentrations ($10^{-8}-10^{-6}$ M) of all agonists were shallow. These data suggest preclusion of an expression of displacement potencies in terms of a single concentration causing 50% displacement of total binding. For the purpose of the present text we conclude that low nanomolar concentrations of the dopamine agonists effectively displace the binding of 2 nM [^{3}H]ADTN. Noradrenaline was approximately 10 times less potent in displacing [^{3}H]ADTN than dopamine, and isoapomorphine and catechol were essentially ineffective at up to 10^{-7} M (results not shown).

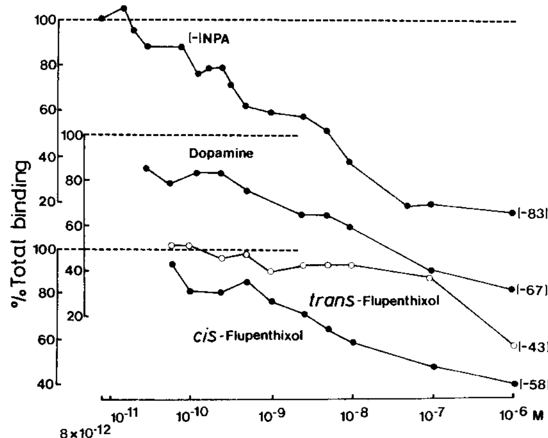

FIG. 4. The abilities of dopamine agonists and antagonists to displace the binding of 2 nM [^{3}H]ADTN. % values given on the right hand side indicate the displacements caused by 10^{-6} M of the compounds tested. All assays were carried out in the presence of freshly prepared ascorbic acid, 10^{-4} M. n = 4–8. s.e.m.s <3%.

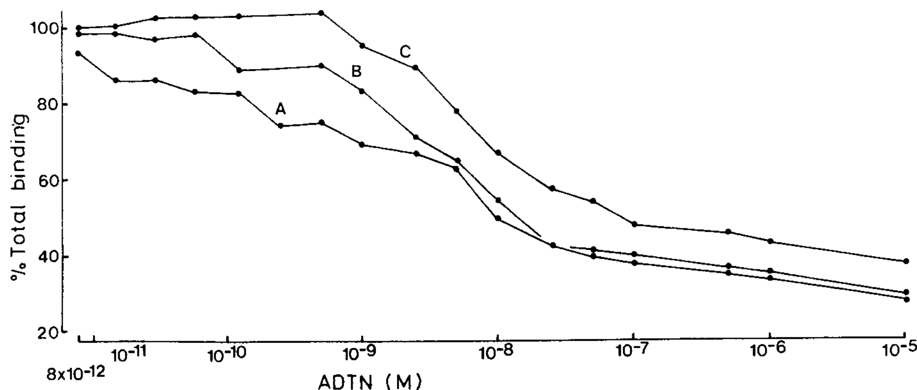

FIG. 5. The effect of membrane ageing on the ability of (±)-ADTN to displace the binding of 2nM [^{3}H]ADTN. A membranes freshly prepared, B membranes stored on ice for 1 h or C 2 h. n = 4–8. s.e.m.s on original data <4%. Values indicated in A and B show a significant difference from corresponding values in C over the range $1{\cdot}6 \times 10^{-11}$–$10^{-5}$ M and 10^{-10}–10^{-5} M respectively ($P < 0{\cdot}001$, Student's *t*-test).

cis-Flupenthixol significantly displaced [^{3}H]-ADTN binding by approximately 20% at 6×10^{-11}–10^{-9} M, increasing to 60% at 10^{-6} M. In contrast, *trans*-flupenthixol caused a maximal displacement of only 10% at concentrations up to and including 10^{-7} M (Fig. 4). Fluphenazine and (+)-butaclamol had similar actions and potencies to *cis*-flupenthixol, domperidone and haloperidol displaced [^{3}H]ADTN in concentrations higher than 5×10^{-10} M, causing a 40% reduction in total binding at 10^{-6} M, whilst metoclopramide (10^{-10}–10^{-6} M) failed to significantly displace [^{3}H]ADTN binding. (+)- and (−)-sulpiride had indistinguishable effects, that is, slight but consistent trends to displace [^{3}H]ADTN binding only approaching 20% at 10^{-6} M and propranolol, prazosin and yohimbine (10^{-11}–10^{-5} M) failed to modify [^{3}H]ADTN binding to rat striatal membranes (results not shown).

VI. *The effect of membrane ageing on [^{3}H]ADTN binding*

A rat striatal membrane suspension was prepared in Tris-HCl buffer containing no ascorbic acid and maintained on ice with gentle agitation. Samples were taken immediately or after 1 and 2 h for incubation with 2 nM [^{3}H]ADTN (freshly prepared ascorbic acid, 10^{-4} M, was added to the assay tubes, with (±)-ADTN as displacing agent, 8×10^{-12}–10^{-5} M, immediately before addition of the ligand). When membranes were used immediately on completion of their preparation, (±)-ADTN displaced total binding by approximately 15% in the concentration range 8×10^{-12}–$1{\cdot}25 \times 10^{-10}$ M, with increasing displacement following increasing (±)-ADTN concentration. The phase of displacement occurring between 8×10^{-12}–$1{\cdot}25 \times 10^{-10}$ M (±)-ADTN was either absent or reduced when membranes were used after a 1 h delay, although subsequent displacements were similar to those recorded using the freshly prepared membranes. When membranes were maintained on ice for 2 h before use the lower concentrations of (±)-ADTN (8×10^{-12}–5×10^{-10} M) failed to displace [^{3}H]ADTN binding; displacement did occur at higher concentrations of (±)-ADTN but this was always less than observed using the other membrane preparations, the final displacement at 10^{-5} M being 10% less (Fig. 5).

DISCUSSION

Scatchard analyses carried out in the absence of ascorbic acid show a saturable (B_{max} 138 fmol mg^{-1} protein) and high affinity (K_D 1·26 nM) binding for [^{3}H]ADTN to rat striatal membranes, but the variations about the mean values used to construct these plots were large. These variations were dramatically reduced when assays were carried out in the presence of ascorbic acid, 10^{-4} M or 5·7 mM. This 'regularization' of binding, also noted by List & Seeman (1982), is not understood, but is, nevertheless, a most consistent observation. However, the major finding of the present study is that the binding of [^{3}H]ADTN to rat striatal membranes can be reduced by ascorbic acid with a critical dependence not only on the concentration of ascorbic acid used but also on the age of the ascorbic acid solution. Ascorbic acid 10^{-4} M was selected, as in preliminary experiments this was the maximal concentration that could be used immediately on preparation to regularize [^{3}H]ADTN binding without changing the ligand's characteristics. The design of subsequent

experiments, such as those involving Scatchard analyses, demanded the use of large sample numbers which necessarily prolonged the preparatory steps for up to 15 min. When the ascorbic acid solution was so 'aged', there was a modest but significant decrease in total and 'specific' (dopamine/*cis*-flupenthixol displaceable) [^{3}H]ADTN binding, although the binding sites had slightly increased affinity (K_D 0·98 nM). In contrast, if the preparatory steps allowed for a 60 min delay in the use of ascorbic acid, affinity was slightly reduced. Nevertheless, a comparison of these effects of 10^{-4} M ascorbic acid with those of 5.7 mM emphasizes the deleterious effects of the latter, a concentration widely used in binding studies and which, when used after only 15 min, caused a marked reduction in total binding of [^{3}H]ADTN (0.25–4.0 nM) and abolished all binding of 0·125 nM [^{3}H]ADTN. Of the [^{3}H]ADTN binding that remained in the presence of ascorbic acid, approximately 70% was displaceable by dopamine but a Scatchard projection indicated such binding to be of markedly lower affinity (K_D 7.2 nM) with a greater number of binding sites (207 fmol mg^{-1} protein). The use of a 5·7 mM) solution of ascorbic acid prepared for 60 min also enhanced the number of [^{3}H]ADTN binding sites yet reduced the affinity of [^{3}H]ADTN for the receptor.

These analyses of the effects of ascorbic acid on the binding of [^{3}H]ADTN to rat striatal membranes have employed the classical approach of Scatchard analysis. We accept the limitations of this approach (Klotz 1982). However, it remains an unequivocal finding that 5·7 mM ascorbic acid, used either as a fresh solution or after 60 min, caused a clear reduction in the total binding of [^{3}H]ADTN and drastically reduced the affinity of [^{3}H]ADTN for its binding sites. That the proportion of 'specific' binding sites is slightly increased by this procedure (see also Leff et al 1981) would appear inconsequential in relation to the observation of a reduced affinity. In contrast, the binding of [^{3}H]ADTN to rat striatal tissue in the presence of a freshly prepared solution of ascorbic acid (10^{-4} M) was characterized by high affinity binding specifically displaceable by nano- and subnanomolar concentrations of dopamine, (−)-NPA, (±)-ADTN and apomorphine, and the dopamine antagonists *cis*-flupenthixol, fluphenazine, haloperidol and domperidone. It is important to emphasize that isoapomorphine and catechol were ineffective in displacing [^{3}H]ADTN, indicating that the binding did not occur to a non-specific, high affinity 'catechol' recognition site, and that *trans*-flupenthixol failed to displace [^{3}H]ADTN, indicating the thioxanthine-[^{3}H]ADTN interaction is stereoselective.

These results have important implications for the design of assay conditions demonstrating the binding of dopamine agonists to rat striatal tissue and may provide an explanation for the many interstudy discrepancies. Whilst it would be preferable to directly and individually compare the present data with those obtained by other workers (see the introduction) the differences in assay conditions with respect to temperature, time of incubation, pH, ion concentrations, presence of Na_2EDTA, ascorbic acid concentrations, membrane preparation etc. make this impossible, but a few general points remain immediately obvious. It is virtually certain that different groups will prepare the buffer solutions at different times, maintain solutions at different temperatures, incorporate ascorbic acid at different times and that the use of these buffer solutions, whilst ostensibly having consistent characteristics have a different 'age', a critical and determining factor demonstrated by the present work. There does not exist a single paper in the literature where the age of the buffer can be established, to preclude any firm conclusions with respect to a comparison between the various studies. A further complication is the use of other buffer additives, for example, Na_2EDTA is a variable constituent and this may moderate the deleterious effects of ascorbic acid (Heikkila et al 1982; see also Blaug & Hajratwala 1972). Nevertheless, it is interesting that Chan et al (1982) found that a preincubation with ascorbic acid produced a 'profound time dependent decline in [^{3}H]spiperone binding to canine striatal receptors', although it is not clear whether the decline was due to a progressive membrane ageing or to membrane damage caused by the increasingly toxic action of ascorbic acid. It remains directly relevant that ascorbate had 'minimal effect' on [^{3}H]spiperone binding if introduced simultaneously with the [^{3}H]spiperone; an analogy with the present results is obvious.

It is assumed that ascorbic acid was initially included in assay buffer systems to reduce oxidation of sensitive substances such as catecholamines (Leff et al 1981), and it may perform this function for some ligands (see Arana et al 1982), but an additional and unwanted action is a direct effect on membrane preparations by reducing the binding of many ligands (Leslie et al 1980; Dunlap et al 1979). This may involve ascorbate catalysed lipid peroxidation (Schaefer et al 1975; Heikkila et al 1982).

The present studies were not designed to exhaus-

tively examine all the many permutations in assay conditions but to investigate the consequences of including ascorbic acid in the buffer medium and to identify a methodology for obtaining a 'reasonable' binding. However, from the present studies there emerged a further factor which can powerfully influence [^{3}H]ADTN binding, that is, membrane-receptor stability. Whilst it is perhaps a rather obvious point that membrane deterioration occurs from the moment of tissue removal from the animal, the degree of deterioration has not been reported and is impossible to assess from the published assay methodologies. The present studies show that [^{3}H]ADTN binding to rat striatal tissue can have different spectra depending on whether membranes are used immediately on preparation or after 1 or 2 h. Whilst the use of membranes from all three times indicated a binding displaceable by 5×10^{-10}–2×10^{-8} M (±)-ADTN (and neuroleptics, unpublished data) and very shallow displacement curves at 2×10^{-8}–10^{-5} M, membranes that were used immediately after preparation demonstrated additional binding sites from which [^{3}H]ADTN was displaceable by picomolar concentrations of dopamine agonists, fluphenazine, (+)-butaclamol and *cis*-flupenthixol. These very high affinity sites were less evident 1 h after preparation and could not be detected after 2 h. These results are primarily included to emphasize the importance of membrane deterioration, and a further source of inter-laboratory variation, for it is unlikely that these very high affinity binding sites could have been detected in earlier studies using prolonged membrane preparatory steps. For example, 'storage of brains in ice for 1 to 2 h after death before dissection and homogenate preparation' (List et al 1982) may account in this study for the absence of the very high affinity sites shown in the present experiments. A more precise analysis of the picomolar-affinity binding sites indicated by the present studies is being undertaken, and their significance, if any, assessed. Until a full characterization of these sites is available, and can be confirmed in other laboratories, it would be imprudent to make further difficulties to the challenges of nomenclature of the different dopamine receptor binding sites (see Seeman 1982; Creese & Sibley 1982) by referring to them other than as 'high affinity' sites.

In conclusion, it is emphasised that whilst, so far, comment can only be made with regard to the binding of [^{3}H]ADTN to rat striatal tissue, it is clear that the age of the ascorbic acid solution and the age of the striatal membranes used in the assay procedures can critically determine the characteristics of the [^{3}H]ADTN binding. The indeterminate nature of these two parameters in all previously published work precludes meaningful inter-study comparisons. It is suggested that the precise preparation and use of ascorbic acid and membranes should be clearly stated in future studies to allow a replication of methodology and thus verification of experimental findings in different laboratories.

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