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# Release of the antioxidants ascorbate and urate from a nitrergically-innervated smooth muscle

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**1** The main object of the present study was to determine whether ascorbate, an antioxidant which has been shown to protect nitric oxide (NO) from attack by scavenger molecules, might be released from nitrergically-innervated smooth muscle; ascorbate release from the rat anococcygeus was measured by use of h.p.l.c. with electrochemical detection.

**2** Incubation of rat anococcygeus muscles in normal physiological salt solution (PSS; 30 min) resulted in release of ascorbate into the bathing medium ($7.7 \pm 0.9$ nmol $g^{-1}$ tissue). This release was increased by 96% when muscles were incubated in high $K^+$ (70 mM) PSS. The resting release of ascorbate was unaffected by tetrodotoxin (TTX; 1 $\mu$M), $\omega$-conotoxin GVIA ( 10 nM) or omission of calcium ions from the PSS (with addition of 0.2 mM EGTA), but all three procedures attenuated the increased release observed under depolarizing conditions. Resting release of ascorbate was unaffected by glutamate (100 $\mu$M), aspartate (100 $\mu$M), $\gamma$-aminobutyric acid (100 $\mu$M) or carbachol (50 $\mu$M).

**3** A second h.p.l.c. peak, which always preceded the ascorbate peak, was identified as urate. Urate release from the anococcygeus, following 30 min incubation in normal PSS, was $64.6 \pm 12.7$ nmol $g^{-1}$ tissue but, unlike ascorbate, urate release was unchanged in high $K^+$ PSS. In functional experiments, urate (100–400 $\mu$M) partially protected NO (15 $\mu$M)-induced relaxations of the rat anococcygeus from inhibition by 2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide (carboxy-PTIO; 50 $\mu$M), but not from inhibition by hydroquinone or duroquinone (both 100 $\mu$M).

**4** Muscles chemically sympathectomized with 6-hydroxydopamine (6-OHDA, 500 $\mu$M; 2 h) still exhibited release of ascorbate ($2.5 \pm 0.4$ nmol $g^{-1}$ tissue) and urate ($22.2 \pm 2.9$ nmol $g^{-1}$ tissue); in both cases the release was similar to that observed in time-matched control tissues not exposed to 6-OHDA. High $K^+$ PSS produced a TTX-sensitive increase in release of ascorbate, but not urate, from 6-OHDA-treated muscles.

**5** The results demonstrate that significant amounts of ascorbate and urate are released from the rat anococcygeus muscle. Ascorbate, but not urate, release appears to be enhanced by activation of nerves which are resistant to 6-OHDA pretreatment. Since both antioxidants can protect NO from attack by scavenger molecules, their release in nitrergically-innervated tissues may be important for the provision of the correct redox environment to allow NO to fulfill its proposed neurotransmitter role.

**Keywords:** Anococcygeus (rat); antioxidants; ascorbate; $\omega$-conotoxin; depolarization; free radical scavengers; 6-hydroxydopamine; nitric oxide; tetrodotoxin; urate

## Introduction

It is now clear that many examples of non-adrenergic, non-cholinergic (NANC) relaxations of respiratory, gastrointestinal and urogenital smooth muscle are mediated by so-called nitrergic nerves, which utilize nitric oxide synthase for the generation of their neurotransmitter (Rand & Li, 1995a,b). This novel concept of NO as a neurotransmitter has challenged several of the existing dogma relating to the mechanisms underlying neurotransmission. For instance, NO is not stored within the nerve, but is synthesized on demand and release is by simple diffusion rather than by vesicular stimulus-secretion coupling. In addition, the criterion of mimicry has been challenged by observations that certain drugs could inhibit relaxations of smooth muscle to exogenous NO, but were without effect on nitrergic relaxations in the same tissues (Gibson *et al*., 1995; Rand & Li, 1995a,b). For example, in the rodent anococcygeus muscle, nitrergic relaxations were resistant to inhibition by superoxide anion generators and direct NO-scavengers (Hobbs *et al*., 1991; Rajayanagam *et al*., 1993; Rand & Li, 1995c), which were potent inhibitors of relaxations to exogenous NO. A partial explanation for this paradox has been provided by observations that free radical NO released from the nitrergic nerves is protected from superoxide anions by the presence of copper/zinc superoxide dismutase (Cu/Zn SOD) in the neuroeffector region; exogenous NO, on the other hand, is vulnerable to attack while in the bathing medium, before reaching the tissue (Martin *et al*., 1994; Lilley & Gibson, 1995; Paisley & Martin, 1996; Lefebvre, 1996; De Man *et al*., 1996). However, tissue Cu/Zn SOD activity cannot explain the resistance of nitrergic relaxations to direct NO-scavengers, which do not act via the generation of superoxide anions, although the general principle of the existence of 'NO-minder' molecules within the tissue may still be applicable.

Several other antioxidants, apart from Cu/Zn SOD, can protect NO; in particular, ascorbate has been shown to protect exogenous NO from attack by superoxide anions, hydroquinone and carboxy PTIO (Lilley & Gibson, 1996). This protective effect of ascorbate is not shown by its oxidized form, dehydroascorbic acid (DHA; Lilley & Gibson, 1997a). In the brain, ascorbate acts as an important extracellular antioxidant (Frei, 1994) and its concentration in the extracellular fluid is strictly maintained between 200–400 $\mu$M (Schenk *et al*., 1982; Fillenz *et al*., 1986; Grunewald, 1993). In addition, there is evidence that it may be released from central neurones, either via an amino acid/ascorbate hetero-exchange mechanism or by depolarization (Cammack *et al*., 1991; Grunewald, 1993; Walker *et al*., 1995), and this release may be important in maintaining the extracellular antioxidant concentrations. In the periphery, ascorbate is taken up, and released, by adrenal chromaffin cells (Daniels *et al*., 1982; Diliberto *et al*., 1983), and it is known to be an important co-factor for the hydro-

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xylation of dopamine in sympathetic nerves (Kaufman & Friedman, 1965). However, the relationship between ascorbate and peripheral nitrergic nerves has not been investigated. The object of the present study was to determine whether ascorbate might be released from the rat anococcygeus, a nitrergically-innervated smooth muscle, since such release would be necessary if ascorbate was to be considered as a possible protector of neurotransmitter NO.

During the course of the study it became clear that urate, another important extracellular antioxidant (Ames *et al*., 1981; Frei, 1994), was also released from the rat anococcygeus, and therefore results of experiments with this substance are included. Some of the work has been presented to the British Pharmacological Society (Lilley & Gibson, 1997a,b).

## Methods

### *Rat anococcygeus muscles* in vitro

Male rats (Wistar; 250–350 g) were killed by stunning and exsanguination. The anococcygeus muscles (Gillespie, 1972) were dissected and set up in 2 ml glass organ baths containing physiological saline solution (PSS; mM: NaCl 118.1, KCl 4.7, $MgSO_4$ 1.0, $KH_2PO_4$ 1.0, $CaCl_2$ 2.5, $NaHCO_3$ 25.0 and glucose 11.1) which was maintained at 37°C and gassed continuously with 95% $O_2$: 5% $CO_2$. The tissues were set up with a resting tension of 0.8–1.0 g and changes in tension were monitored with a Grass FTO3 force displacement transducer attached to a Graphtech pen-recorder (WR 3101). A period of 45 min was allowed before the start of the experiment, for tissue equilibration. Field stimulation was applied to the tissue via two parallel platinum electrodes running down either side of the tissue; these were attached to a Grass S48 stimulator (0.5 ms pulse width; 70 V). Sympathetic responses were negated by including 1 $\mu$M phentolamine in the PSS and by pre-incubating each muscle with 30 $\mu$M guanethidine for 15 min during the equilibration time.

In experiments where sympathetic neurones were destroyed with 6-hydroxydopamine (6-OHDA), rat anococcygeus muscles were set up as detailed above. After 45 min equilibration time, tissues were exposed to field stimulation (20 Hz; 10 s train every 100 s) for 10 min to record sympathetic contractions. Tissues were then treated with 500 $\mu$M 6-OHDA for 120 min (washing out and replacing the drug every 30 min), followed by 10 min washout. Tissues were then tested as above for sympathetic responses to field stimulation. Nitrergic relaxations were examined by raising the tone of the tissues with 50 $\mu$M carbachol and then stimulating at 10 Hz (10 s train every 100 s; Lilley & Gibson, 1996). In some tissues, contractile responses to noradrenaline (1 and 2 $\mu$M) were obtained both before and after exposure to 6-OHDA. Time matched controls were carried out with tissues treated as above, but with the exclusion of 6-OHDA treatment. This method of *in vitro* chemical sympathectomy is a modification of the method described by Doggrell & Waldron (1982). In some experiments, the effects of drugs on relaxations to authentic NO were investigated; again, tone was raised with 50 $\mu$M carbachol.

### *Ascorbate release from rat anococcygeus muscle*

In experiments where ascorbate release into PSS was measured rat anococcygeus muscles were set up for isometric measurement of changes in tension as detailed above, including exposure to both phentolamine and guanethidine. After 45 min had been allowed for equilibration, tissues were transferred to 1.5 ml Eppendorf tubes containing 100 $\mu$l PSS (of either normal composition, or as described in the Results section) which was gassed with 95% $O_2$; 5% $CO_2$. In all cases, 400 $\mu$M reduced glutathione (GSH) was included to prevent oxidation of any ascorbate released. Tissues were incubated in the PSS for the appropriate time at 37°C. A 50 $\mu$l aliquot of PSS solution was then removed for measurement of ascorbate by high performance liquid chromatography (h.p.l.c.) and the tissues weighed.

### *H.p.l.c. measurement of ascorbate*

Ascorbate concentration was determined by h.p.l.c. with electrochemical detection (Pachla & Kissinger, 1979). Separation was achieved with reverse-phase paired-ion h.p.l.c. with a Nova-Pak $C_{18}$ Radial-Pak column (Waters). The mobile phase consisted of 80 mM sodium acetate buffer (1.0 M stock; pH 4.00), 1 mM tridecylamine (100 mM stock; as the ion-pair reagent) and 15% methanol. The final pH of the mobile phase was 4.5, the sample loop was 20 $\mu$l and the flow rate was 1.0 ml $min^{-1}$. Electrochemical detection of ascorbate was achieved with a LC-4A amperometric detector (Bioanalytical Systems Inc.) set at 0.7 V (vs Ag-AgCl electrode). The ascorbate signal was monitored in graphical form by Mac-Lab. Two methods of identifying the ascorbate peak were used; firstly, the relative retention time when compared to ascorbate standards and, secondly, by adding 10 u $ml^{-1}$ of ascorbate oxidase to samples. Ascorbate concentrations were extrapolated from a standard curve which was linear ($r=0.99$) up to 20 $\mu$M. Reproducibility of the assay was >90% and the limit of detection was 76 nM ascorbate. The urate standard curve was also linear ($r=0.99$) up to 20 $\mu$M; reproducibility was again >90% and the limit of detection was 46 nM urate.

### *Statistics*

Results are expressed as mean ± s.e.mean. Statistical analysis was by Student's $t$ test (unpaired); a probability value of $P<0.05$ was taken to indicate statistical significance.

### *Drugs and reagents*

All drugs were dissolved in distilled water, except uric acid which was dissolved in 0.01% (w/v) NaOH (10 mM stock). Preparation of NO solutions was as described previously (Gibson & Mirzazadeh, 1989). H.p.l.c. reagents were dissolved in ultra-pure distilled water, except tridecylamine which was dissolved in methanol.

Drugs used were (supplied by Sigma unless stated otherwise): $\gamma$-aminobutyric acid (GABA; Calbiochem), ascorbic acid, ascorbate oxidase, L-aspartic acid, carbachol, $\omega$-conotoxin (GVIA), dehydroascorbic acid (DHA; Aldrich), ethylene glygol-*bis*($\beta$-aminoethylether) N,N,N′,N′-tetraacetic acid (EGTA), L-glutamic acid, glutathione (GSH), guanethidine sulphate, 6-hydroxydopamine HBr, methanol h.p.l.c.-grade; Rathburn), nitric oxide (BDH), phentolamine HCl, tetrodotoxin, tridecylamine (Aldrich), uric acid, uricase.

## Results

### *Release of ascorbate from control tissues*

H.p.l.c. assay of PSS in which rat anococcygeus muscles had been incubated for 10 min or more resulted in the appearance of two distinct peaks (Figure 1); the second peak was identified as ascorbate by comparison of its retention time with that of ascorbate standards, and by its disappearance on incubation with ascorbate oxidase (Figure 1). The first peak, which was always present, was not reduced by ascorbate oxidase and subsequent experiments showed it to be urate (see later). Neither 400 $\mu$M GSH (included to reduce oxidation of ascorbate) nor 100 $\mu$M DHA (the oxidized form of ascorbate) gave any detectable peaks on the h.p.l.c. Examination of the time-course of the resting release of ascorbate showed that it was almost linear over a 2 h incubation period (Figure 2); 30 min was chosen as a standard incubation time for subsequent experiments.

In normal PSS, the resting release of ascorbate over a 30 min incubation period was 7.7 ± 0.9 nmol $g^{-1}$ tissue ($n=8$).

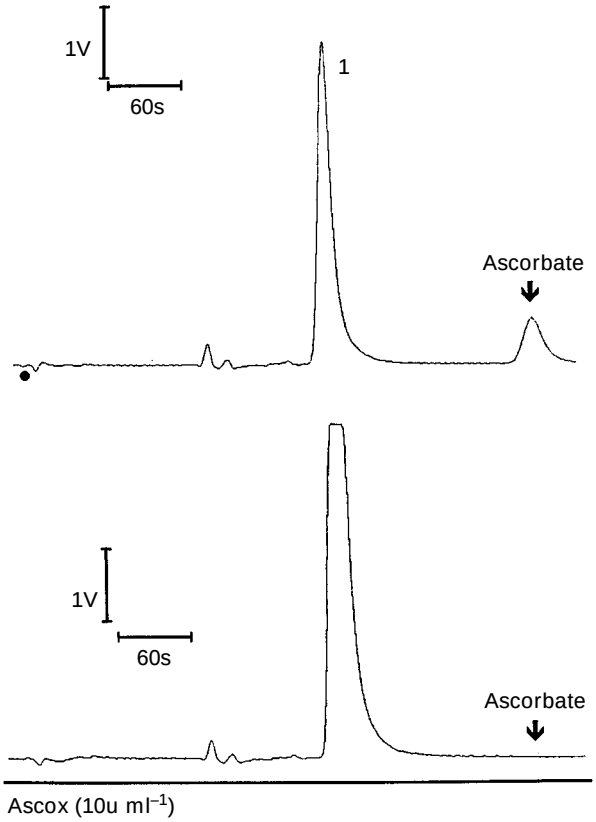


**Figure 1** H.p.l.c. traces showing the peaks obtained following injection (at the dot) of 50 $\mu$l of physiological saline solution in which a rat anococcygeus muscle had been incubated for 30 min, and the selective disappearance of the second (ascorbate) peak on incubation of the same sample with ascorbate oxidase (Ascox). The small deflection between the injection-point and peak 1 represents the solvent front. Peak 1 was later identified as urate (see text and Figure 4).

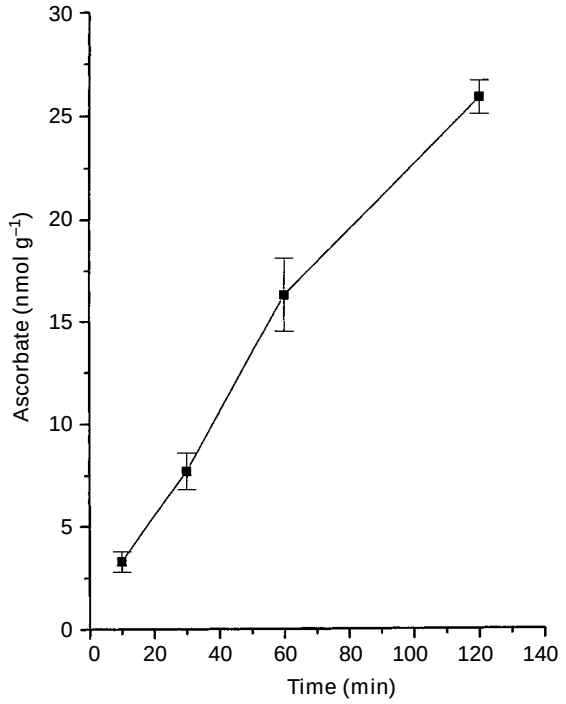


**Figure 2** The amount of ascorbate released from rat anococcygeus muscles incubated in physiological saline solution for different times. Each point is mean from at least five individual muscle preparations; vertical lines show s.e.mean.

In PSS containing high $K^+$ (70 mM with equivalent reduction in $Na^+$), this release was significantly increased by 96% (Figure 3). Inclusion of 1 $\mu$M tetrodotoxin (TTX) in normal PSS, or omission of calcium ions from the bathing medium (plus 0.2 mM EGTA), had no effect on resting release of ascorbate. However, both treatments did abolish the increase in ascorbate release observed in high $K^+$ medium (Figure 3). High $K^+$ failed to produce a significant increase in ascorbate release in the presence of $\omega$-conotoxin GVIA (20 nM; control release $6.4 \pm 1.1$ nmol g$^{-1}$; high $K^+$ release $10.3 \pm 3.0$ nmol g$^{-1}$; $n=4$ in each case; $P>0.05$).

In the brain, there is evidence that basal ascorbate release may be enhanced by amino acids including glutamate, aspartate and GABA (Fillenz *et al*., 1986; Grunewald, 1993), and in the stomach carbachol greatly increases resting ascorbate release (Muto *et al*., 1997). However, resting release of ascorbate from rat anococcygeus was unaffected by either the amino acids (all at 100 $\mu$M; Table 1) or by carbachol (50 $\mu$M; Table 1).

### *Identification of the first peak as urate*

As mentioned earlier, the ascorbate peak was always preceded by a larger h.p.l.c. signal which was resistant to ascorbate oxidase (Figure 1). It is known that urate, another important extracellular antioxidant (Frei, 1994), co-elutes close to ascorbate in h.p.l.c. assays (Iriyama *et al*., 1986; Chevion *et al*., 1997). Indeed, the first peak observed in the present study was

**Table 1** Ascorbate release from rat anococcygeus in the presence of amino acids and carbachol

| *Treatment* | *Ascorbate release* (nmol g$^{-1}$ 30 min$^{-1}$) |
|---|---|
| Control | $7.8 \pm 2.5$ |
| Glutamate (100 $\mu$M) | $6.9 \pm 2.1$ |
| Control | $8.0 \pm 0.8$ |
| $\gamma$-Aminobutyric acid (100 $\mu$M) | $8.7 \pm 0.9$ |
| Control | $9.5 \pm 1.9$ |
| Aspartate (100 $\mu$M) | $9.6 \pm 1.7$ |
| Control | $9.8 \pm 1.5$ |
| Carbachol (50 $\mu$M) | $9.4 \pm 1.4$ |

Values are mean$\pm$s.e.mean. In all cases $n=4$ and $P>0.05$ (treated vs control).

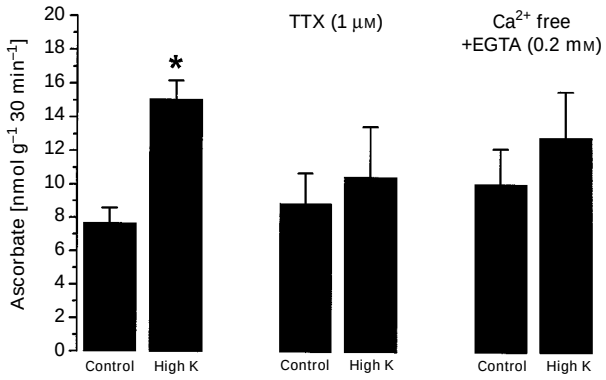


**Figure 3** The amount of ascorbate released from rat anococcygeus muscles incubated for 30 min in physiological saline solution containing normal (5.7 mM; control) or elevated (70 mM; high K) amounts of $K^+$. The middle columns were obtained in the presence of tetrodotoxin (TTX) and the right hand columns in the absence of $Ca^{2+}$ (with 0.2 mM EGTA). Each column is mean$\pm$s.e.mean from at least five individual muscle preparations. *$P<0.05$—significantly different from adjacent control column.

identified as urate since it showed an identical retention time to that of standard urate solutions, and it was abolished in the presence of uricase (100 mu $ml^{-1}$), which had no effect on the ascorbate peak (Figure 4). Resting release of urate following incubation of rat anococcygeus muscles in PSS for 30 min was $65 \pm 13$ nmol $g^{-1}$ tissue ($n=7$); unlike ascorbate, urate release was unchanged in high $K^+$ PSS ($77 \pm 9$ nmol $g^{-1}$ tissue; $n=7$; $P>0.05$).

### *Urate on NO-induced relaxations of the rat anococcygeus*

Previous work has shown that ascorbate can protect NO (15 $\mu$M)-induced relaxations of the mouse anococcygeus from inhibition by duroquinone (superoxide anion generator), hydroquinone (scavenger) and carboxy-PTIO (2-(4-carboxyphenyl)-4,4,5,5-tetramethylimidazoline-1-oxyl-3-oxide, scavenger; Lilley & Gibson, 1996). Experiments were therefore carried out to determine the potential protective effect of urate in the rat anococcygeus, by use of protocols and inhibitor concentrations similar to those used previously (Lilley & Gibson, 1996).

NO 15 $\mu$M relaxed carbachol (50 $\mu$M)-induced tone by $42 \pm 12\%$ ($n=4$) and this was unaffected in the presence of 400 $\mu$M urate ($44 \pm 9\%$; $n=4$; $P>0.05$). However, NO-induced relaxations were inhibited by 100 $\mu$M hydroquinone ($75 \pm 6\%$ inhibition; $n=7$), 100 $\mu$M duroquinone ($44 \pm 6\%$ inhibition; $n=9$) and 50 $\mu$M carboxy-PTIO ($77 \pm 6\%$ inhibition; $n=11$). Urate reduced the inhibitory effect of carboxy-PTIO ($25 \pm 9\%$ reduction and $43 \pm 8\%$ reduction with 100 $\mu$M and 400 $\mu$M urate, respectively; $n=10$ in both cases), but provided no protection against the inhibitory effects of duroquinone or hydroquinone (data not given).

### *Functional response and ascorbate/urate release following 6-OHDA pretreatment*

The preceding results suggested that ascorbate, but not urate, might be released following nerve-activation. Ascorbate is an important co-factor for dopamine-$\beta$-hydroxylase in sympathetic nerves (Kaufman & Friedman, 1965). Since the rat anococcygeus contains a dense noradrenergic innervation (Gillespie, 1980) it was important to determine whether the released ascorbate was coming from these nerves. Release experiments were therefore carried out following chemical sympathectomy of the anococcygeus with 6-OHDA (Doggrell & Waldron, 1982).

Traces from a typical denervation experiment are shown in Figure 5. Before the addition of 6-OHDA, field stimulation (20 Hz; 10 s trains) produced contraction, as did noradrenaline (1 and 2 $\mu$M). 6-OHDA (500 $\mu$M; 120 min with washout and replacement every 30 min) produced a strong, but transient, contraction. After washout of 6-OHDA (10 min), contractions to field stimulation were abolished, while those to noradrenaline were greatly enhanced; this enhancement would be expected due to destruction of the sympathetic nerve terminals and therefore less inactivation of added noradrenaline via neuronal uptake. When tone was raised with carbachol (50 $\mu$M) field stimulation now produced clear relaxations. Al-

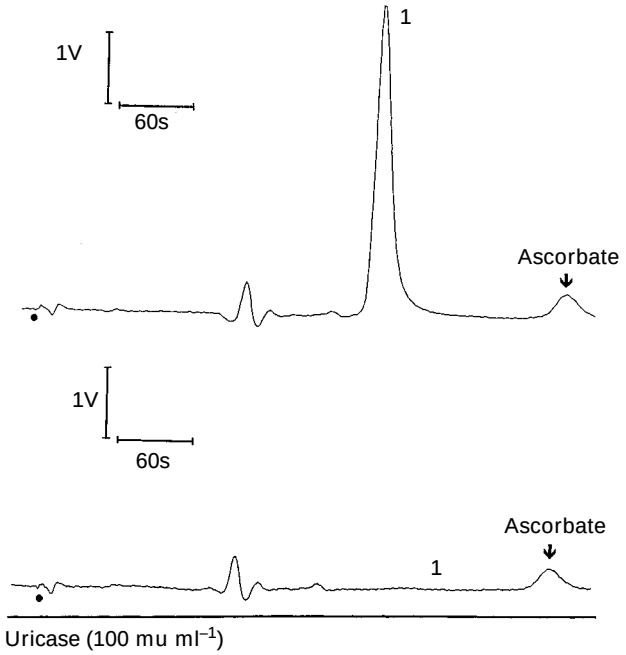


**Figure 4** H.p.l.c. traces showing the peaks obtained following injection (at the dot) of 50 $\mu$l physiological saline solution in which a rat anococcygeus muscle had been incubated for 30 min, and the selective disappearance of the first peak on incubation of the same sample with uricase. The small deflection between the injection-point and peak 1 represents the solvent front.

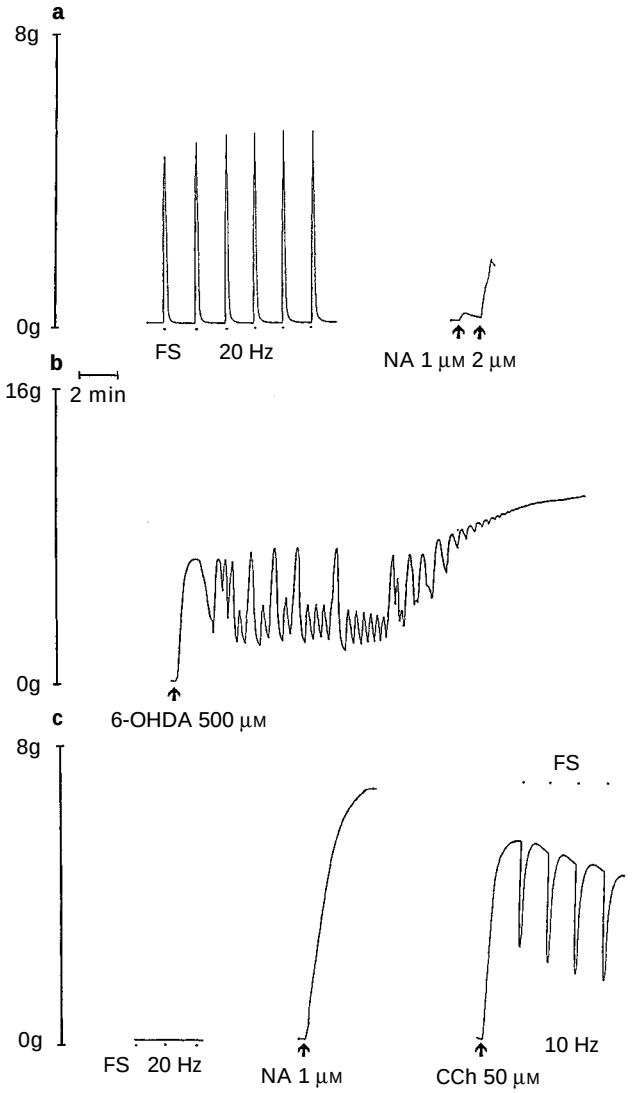


**Figure 5** Traces showing mechanical responses of a rat anococcygeus muscle to field stimulation (FS) and drugs during a typical chemical sympathectomy procedure with 6-hydroxydopamine (6-OHDA). (a) Contractile responses to FS (20 Hz; 10 s trains) and noradrenaline (NA) before incubation with 6-OHDA. (b) Initial contractile response to 6-OHDA. (c) Following 2 h incubation with 6-OHDA (and 10 min washout), sympathetic contractile responses to FS were absent, responses to NA were greatly enhanced, and FS (10 Hz; 10 s trains) caused clear NANC relaxations of carbachol CCh-induced tone.

though not shown, normal relaxations to exogenous NO (15 $\mu$M) were also obtained after 6-OHDA pretreatment.

Resting ascorbate release in time-matched control tissues, not exposed to 6-OHDA, was only 33% of that in the earlier control muscles. However, this release was still significantly increased in high $K^+$ PSS (Figure 6). Resting release in 6-OHDA-treated tissues was similar to that in time-matched controls and again was increased by high $K^+$ (Figure 6). Tetrodotoxin (TTX; 1 $\mu$M) had no effect on the resting release of ascorbate in 6-OHDA-treated tissues, but it prevented the enhanced release observed in high $K^+$ solution (Figure 6).

In 6-OHDA-treated muscles, resting release of urate was $22 \pm 3$ nmol $g^{-1}$ tissue ($n=6$); this was similar to the release obtained from time-matched controls ($26 \pm 5$ nmol $g^{-1}$ tissue; $n=6$), and was unchanged in high $K^+$ PSS ($27 \pm 6$ nmol $g^{-1}$ tissue; $n=6$).

## Discussion

We have previously shown that several physiological antioxidants (Cu/Zn SOD; ascorbate; GSH; $\alpha$-tocopherol) can protect NO from attack by superoxide anions and other scavenging agents (Lilley & Gibson, 1996). Of the antioxidants investigated, ascorbate provided the most wide-ranging protection. Therefore, the object of the present study was to determine whether ascorbate might be released from a nitrergically-innervated tissue, and thereby be a potential endogenous protector of neurotransmitter NO. With the rat anococcygeus as the model tissue, three main findings have emerged. First, ascorbate is indeed released by the rat anococcygeus; secondly, this release is enhanced under depolarizing conditions; and thirdly urate, another antioxidant, is also released by the rat anococcygeus, although in this case the release was not enhanced by depolarization.

Ascorbate is known to be one of the most important of the extracellular antioxidants (Gutteridge & Halliwell, 1994; Frei, 1994) and there already exists considerable evidence linking it with nerve function. In the central nervous system, various transport mechanisms exist to maintain a high ascorbate concentration, both extracellularly (200–400 $\mu$M) and intracellularly (1–2 mM; Schenk *et al*., 1982; Fillenz *et al*., 1986); the extracellular concentration of ascorbate is strictly regulated, probably at the expense of intracellular levels (Schenk *et al*., 1982). In addition, during periods of vitamin deficiency, ascorbate concentrations in the brain are maintained for much longer than in other tissues (Pelletier, 1969). The precise roles of ascorbate within the brain have yet to be fully elucidated but the antioxidant has been shown to modulate several neuronal functions, both at the cellular and behavioural levels (Grunewald, 1993). Despite this wealth of evidence in the central nervous system, little is known about the interaction of ascorbate with peripheral nerves. However, in the present study, several observations suggest that the enhanced release of ascorbate from the rat anococcygeus under depolarizing conditions was associated with neuronal activation, since the enhanced release was reduced by TTX, by $\omega$-conotoxin and by omission of calcium ions from the bathing medium. At present, it is not possible to identify the cellular source(s) of the ascorbate released by high $K^+$, whether directly from nerve terminals or indirectly from some non-neuronal source stimulated by a released transmitter. However, a number of transmitter systems which have been shown to activate ascorbate release in other tissues can be ruled out; in the anococcygeus, basal ascorbate release was not enhanced by amino acids, as has been observed in the brain (Cammack *et al*., 1991; Grunewald, 1993; Walker *et al*., 1995), or by muscarinic agonists such as carbachol, as found in the rat stomach (Muto *et al*., 1997). Neither does it appear that the sympathetic nerves of the anococcygeus (Gillespie, 1980) are involved in the release of ascorbate, either under basal conditions or in depolarizing medium. Ascorbate is an important cofactor for the conversion of dopamine to noradrenaline by dopamine-$\beta$-hydroxylase in sympathetic nerves (Kaufman & Friedman, 1965) and it is taken up and released by adrenal chromaffin cells (Daniels *et al*., 1982; Diliberto *et al*., 1983). However, in the present study, ascorbate release from the anococcygeus was not reduced by 6-OHDA pretreatment when compared with time-matched controls although, as found by Doggrell & Waldron (1982), 6-OHDA successfully blocked sympathetic function without affecting nitrergic relaxations. This finding is similar to results obtained in the brain where, again, 6-OHDA pretreatment failed to affect basal or $K^+$-stimulated release of ascorbate (Grunewald, 1993). Since the other major innervation of the rat anococcygeus is nitrergic, it may be that some, or all of the released ascorbate is derived from these nerves. The ability of TTX to inhibit $K^+$-induced ascorbate release is of interest, since it has previously been shown that $K^+$ can activate release of the non-adrenergic, non-cholinergic transmitter in the rat anococcygeus and thereby cause relaxation. This effect occurs at lower concentrations of $K^+$ than those which cause contraction and, like ascorbate release, it is inhibited by TTX (Gibson & James, 1977). It is also of interest that TTX, like calcium omission or $\omega$-conotoxin, failed to reduce resting release of ascorbate. This suggests the existence of another mechanism of ascorbate release which is not dependent on neuronal depolarization, but it is not clear whether this release, and that evoked by high $K^+$, would be from the same cellular source.

An important question is whether the concentrations of ascorbate in the extracellular space of the anococcygeus ever reach levels which might protect neurotransmitter NO from scavenger attack. The protective concentrations of ascorbate vary with the attacking agent (100–400 $\mu$M ascorbate is effective against superoxide anions; 50–400 $\mu$M against hydroquinone; 10–200 $\mu$M against carboxy-PTIO; Lilley & Gibson, 1996). As mentioned previously, extracellular concentrations of ascorbate in the brain are maintained between 200–400 $\mu$M, but this is due to the presence of selective uptake processes, both in the choroid plexus and in the neuropil, which concentrate ascorbate in cerebrospinal fluid compared with blood plasma (50 $\mu$M). It is unlikely that such high levels of extracellular ascorbate are maintained in the anococcygeus. The ascorbate content of the rat anococcygeus is some twenty times

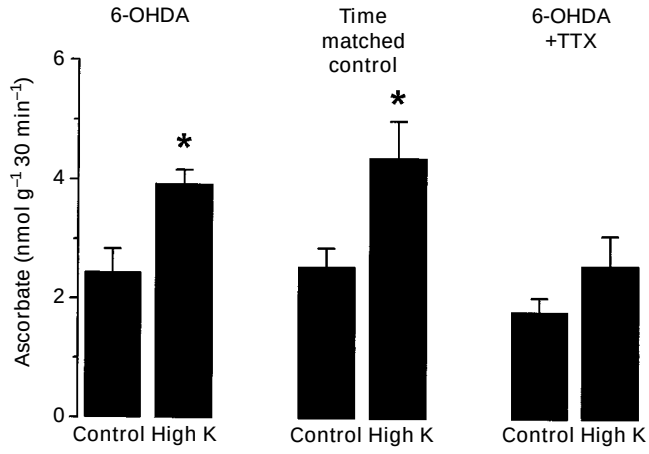


**Figure 6** The amount of ascorbate released from rat anococcygeus muscles incubated for 30 min in physiological saline solution containing normal (5.7 mM; control) or elevated (70 mM; high K) amounts of $K^+$. The two left hand columns were obtained from muscles which had been exposed to 6-hydroxydopamine (6-OHDA) for 2 h (as in Figure 5) and the middle columns from time-matched control muscles, not exposed to 6-OHDA. The right hand columns were obtained from muscles exposed to 6-OHDA, and then to tetrodotoxin (TTX) during the 30 min ascorbate-release incubation. Each column is mean $\pm$ s.e.mean from at least 5 individual muscle preparations. *$P<0.05$—significantly different from adjacent control column.

less than that of rat brain (Lilley & Gibson, 1997a), although if the ascorbate is indeed located in nerves then this may simply reflect the different neuronal densities in the two tissues. Several factors make it difficult to estimate the concentration of ascorbate which might be achieved in the nitrergic neuroeffector junction: the local concentration will be much higher in the vicinity of the releasing cell, especially during nerve activation; several ascorbate uptake processes have been identified (Rose, 1988; Wilson, 1989) which might sequester much of the released ascorbate before it leaks into the bathing medium; and, although the antioxidant GSH was included in the bathing medium, oxidation of ascorbate within the tissue might significantly reduce the amount detected. Experiments in which ascorbate-selective carbon-fibre electrodes are utilized (Cammack *et al*., 1991; Schenk *et al*., 1982; Walker *et al*., 1995), perhaps in a more readily accessible nitrergically-innervated tissue, may help to resolve this issue.

An unexpected finding was that urate was also released by the rat anococcygeus. The basal release of urate was some ten times greater than that of ascorbate but it was not enhanced by depolarization and therefore, unlike that of ascorbate, appears not to be under neuronal control. Urate is also considered to be an important extracellular antioxidant (Ames *et al*., 1981; Frei, 1994; O'Neil & Lowry, 1995) and it is released in brain, possibly from astrocytes (O'Neil & Lowry, 1995). In the present study, urate was found partially to protect exogenous NO from attack by carboxy-PTIO, but not by superoxide anions or hydroquinone; urate must therefore be added to the list of physiological antioxidants (Cu/Zn SOD; ascorbate; GSH; $\alpha$-tocopherol) which have been shown to protect the biological actions of NO. Urate is produced during the metabolism of purines (O'Neil & Lowry, 1995) but, as with ascorbate, it is not possible to determine from the present study either the source of the released urate or the concentrations which might be achieved in the neuroeffector region. Also like ascorbate, the resting release of urate was markedly reduced in the time-matched control tissues during the 6-OHDA experiments. This was most likely due to the prolonged incubation time in these experiments (about 3 h before measuring release) resulting in some depletion of the antioxidants.

In conclusion, the results of this study have shown that the nitrergically-innervated rat anococcygeus muscle can release two important extracellular antioxidants; the release of ascorbate, but not urate, is enhanced by activation of nerves which are resistant to 6-OHDA pretreatment. Since both ascorbate and urate can protect NO from scavenger attack, their release in nitrergically-innervated tissues, probably in conjunction with other antioxidants (Gibson & Lilley, 1997), may be important in providing the correct redox environment to allow NO to fulfill its neurotransmitter role. Important questions still to be answered concern the cellular source of the released antioxidants, and the concentrations of each in the nitrergic neuroeffector junction during nerve-activation.

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