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Uric acid–iron ion complexes

A new aspect of the antioxidant functions of uric acid

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1. In order to survive in an oxygen environment, aerobic organisms have developed numerous mechanisms to protect against oxygen radicals and singlet oxygen. One such mechanism, which appears to have attained particular significance during primate evolution, is the direct scavenging of oxygen radicals, singlet oxygen, oxo-haem oxidants and hydroperoxyl radicals by uric acid. 2. In the present paper we demonstrate that another important 'antioxidant' property of uric acid is the ability to form stable co-ordination complexes with iron ions. Formation of urate–Fe^{3+} complexes dramatically inhibits Fe^{3+}-catalysed ascorbate oxidation, as well as lipid peroxidation in liposomes and rat liver microsomal fraction. In contrast with antioxidant scavenger reactions, the inhibition of ascorbate oxidation and lipid peroxidation provided by urate's ability to bind iron ions does not involve urate oxidation. 3. Association constants (K_a) for urate–iron ion complexes were determined by fluorescence-quenching techniques. The K_a for a 1:1 urate–Fe^{3+} complex was found to be 2.4×10^5, whereas the K_a for a 1:1 urate–Fe^{2+} complex was determined to be 1.9×10^4. Our experiments also revealed that urate can form a 2:1 complex with Fe^{3+} with an association constant for the second urate molecule (K'_a) of approx. 4.5×10^5. From these data we estimate an overall stability constant ($K_s \simeq K_a \times K'_a$) for urate–$Fe^{3+}$ complexes of approx. 1.1×10^{11}. 4. Polarographic measurements revealed that (upon binding) urate decreases the reduction potential for the Fe^{2+}/Fe^{3+} half-reaction from -0.77 V to -0.67 V. Thus urate slightly diminishes the oxidizing potential of Fe^{3+}. 5. The present results provide a mechanistic explanation for our previous report that urate protects ascorbate from oxidation in human blood. The almost saturating concentration of urate normally found in human plasma (up to 0.6 mM) represents 5–10 times the plasma ascorbate concentration, and is orders of magnitude higher than the 'free' iron ion concentration. These considerations point to the physiological significance of our findings.

INTRODUCTION

The importance of oxygen radicals and other activated oxygen species in tissue injury and disease is now widely recognized [1]. Recently, Ames *et al.* [2] proposed that uric acid may act as an important physiological antioxidant defence against such oxidative injuries. The basis for this hypothesis was the observation that uric acid is oxidized to allantoin (and other products) in a process that scavenges hydroxyl radicals ($^{\cdot}OH$), lipid hydroperoxide radicals, singlet molecular oxygen ($^1O_2$), and oxo-haem oxidants, while inhibiting lipid peroxidation. Earlier studies had also reported the ability of urate to scavenge $^1O_2$ [3] and oxo-haem oxidants [4], as well as the protective effect of urate on linoleic acid stability [5] and erythrocyte membrane integrity [6]. During the course of human evolution several mutational events have resulted in the loss of uricase activity (urate → allantoin) and the development of an active kidney re-absorption system for urate. These factors combine to produce blood urate concentrations in man that are some 10 times higher than those found in most other mammals [7]. The accompanying loss of ascorbate synthetase activity during human evolution led Ames *et al.* [2] to propose that uric acid may have replaced some (but not all) of the antioxidant functions of ascorbic acid. A somewhat similar proposal was also made by Proctor [8].

The antioxidant properties of uric acid discussed above involve urate oxidation via classical scavenger mechanisms. During the course of further studies, however, we noted that urate can also inhibit oxidative reactions without itself being oxidized. Specifically, we reported that uric acid appears to be necessary to prevent the oxidation of ascorbic acid in human blood, although no urate oxidation results from this protection [9]. The mechanism by which urate preserved ascorbate was not clear, but several alternatives were discussed. A key observation in the previous paper was the fact that metal-ion-chelating agents such as EDTA and DTPA almost completely inhibited the oxidation of ascorbate in human blood [9]. In the present paper we provide evidence that urate inhibits ascorbate oxidation, and ascorbate-dependent lipid peroxidation, by forming stable complexes with transition-metal ions such as iron ions. Ascorbate oxidation is largely metal-ion-dependent at neutral pH, and we propose that urate–Fe^{3+} complexes are ineffective catalysts of ascorbate oxidation. Our findings add a new dimension to the biological significance of uric acid as a primary 'antioxidant', and

Abbreviation used: DPTA, diethylenetriaminepenta-acetic acid.
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further highlight the connections between urate and ascorbate in human metabolism.

MATERIALS AND METHODS

Ascorbate oxidation

Ascorbate oxidation was monitored spectrophotometrically by absorbance loss at 265 nm ($\epsilon_{265} = 7 \times 10^3$ M^{-1}·cm^{-1}), and polarographically by O_2 consumption with the use of a Clark electrode. Initial rates of oxidation were compared in the presence of various concentrations of ferric iron salts (Fe^{3+}) and/or urate, and were determined over the pH range 6.5–12.0. Concentrations of urate remaining, after 1 min incubation with ascorbate ($+Fe^{3+}$), were determined spectrophotometrically by absorbance at 292 nm, as well as by comparisons of the bimodal urate absorption spectrum in the range 220–320 nm. Urate and ascorbate concentrations after incubation with Fe^{3+} were also monitored by h.p.l.c. with the use of isocratic elution with 1% acetic acid on a 10 μm RP-18 column (Waters Associates).

Ascorbate oxidation was also studied by e.s.r. spectroscopy with a Varian E-3 spectrometer. The basic design of these experiments was to measure the production of the ascorbyl radical (the one-electron oxidation product of dihydroascorbate) during iron-ion-catalysed ascorbate oxidation, in the presence and in the absence of urate (as above).

Lipid peroxidation

The extent of peroxidation after incubation with ascorbate and Fe^{3+} was measured by the thiobarbituric acid method [10] in unilamellar liposomes and rat liver microsomal fraction. Incubations were performed in the presence of various ascorbate and urate concentrations ($+Fe^{3+}$) in a shaking water bath at 37 °C, and the reactions were terminated by addition of trichloroacetic acid.

Unilamellar liposomes were prepared by mixing 50 mg of (bovine liver) phosphatidylcholine/phosphatidylethanolamine (4:1 ratio) in 10 ml of 0.15 M-KCl/10 mM-Tris/HCl buffer, pH 8.0 (saturated with argon). The phospholipid mixture was sonicated [11] under an argon atmosphere, and small unilamellar liposomes were collected as the 100000 *g* supernatant after a 30 min centrifugation. After incubation with ascorbate + Fe^{3+} in the presence and in the absence of urate, butylated hydroxytoluene in 16.8% (v/v) Lubrol PX was added to give a final concentration of 0.5 mM butylated hydroxytoluene and 0.8% Lubrol. This avoids ascorbate-independent Fe^{3+}-catalysed lipid peroxidation [10]. Subsequently, 0.5 vol. of 50% (w/v) trichloroacetic acid and an equal volume of 0.75% thiobarbituric acid in 0.1 M-HCl were added. The samples were boiled for 20 min, and thiobarbituric acid-reacting substances were determined spectrophotometrically at 532 nm. Uric acid had no effect on the thiobarbituric acid test itself under these conditions (or any others so far tested).

Microsomal fraction was isolated from the livers of male Sprague–Dawley rats (200–250 g body wt.) as previously described [12]. Protein was determined by the biuret procedure [13]. After incubation with ascorbate with or without Fe^{3+} in the presence and in the absence of urate, 0.5 vol. of 40% trichloroacetic acid was added and the protein was removed by centrifugation at 7000 *g* for 10 min at 4 °C. The supernatant fractions were then used to determine thiobarbituric acid-reactive substances as described above.

Measurements of urate–iron ion interaction

The iron-ion-binding properties of urate were determined by fluorescence-quenching experiments [14, 15]. Uric acid was dissolved in 100 mM-potassium phosphate buffer, pH 7.0, in the presence or in the absence of various concentrations of $FeCl_3$ or $FeSO_4$. All experiments were conducted under an N_2 atmosphere. The ability of $FeCl_3$ or $FeSO_4$ to quench the fluorescence of urate was measured with an Aminco–Bowman spectrofluorimeter, at 306 nm excitation and 395 nm emission. Suitable buffer blanks were subtracted in all cases. As shown by Weber [14, 15], non-collisional fluorescence quenching may be used to derive the association constant (K_a) of a given complex. In this procedure the concentration of quencher ($FeCl_3$ or $FeSO_4$) is plotted against $I_0/I-1$ (where I_0/I is the ratio of the fluorescence intensities before and after the addition of the quencher) and the slope is used to derive the association constant (K_a). This method requires that the concentration of the quencher exceed that of the ligand, and thus K_a can only be calculated for 1:1 complexes. To examine higher-order complexes we measured I_0/I at various iron ion concentrations, in the presence of an excess of urate. Under these conditions estimates of successive K_a values and overall stability constants (K_s) can be made if the systems exhibit co-operativity in binding.

The electrical reduction potential for Fe^{3+} was measured by using a polarographic half-cell and a stationary platinum working electrode, referenced to a standard calomel electrode. Polarographic methods and precautions were followed as described by Adams [16]. Current–voltage measurements were made with a Princeton Applied Research model 174A polarographic analyser. Reduction potentials were determined in 10 mM-KCl, to which $FeCl_3$ solutions in water were added to give final concentrations ranging from 50 to 200 μM. All measurements were preceded by a 5 min gassing of the test solutions with N_2, and all reagents were originally saturated with N_2. A 10 mV/s scan rate was used over a 1.5 V range starting at 0.9 V. To examine the effect of uric acid on the Fe^{3+} reduction potential a KCl test solution was prepared to contain 500 μM-urate, and $FeCl_3$ was then added as described above. Five successive scans were made for each preparation studied, and the average reduction potentials were calculated from the polarograms.

RESULTS

Iron-ion- and pH-dependence of ascorbate oxidation

The oxidation of ascorbate was highly dependent on both Fe^{3+} concentration and pH (Fig. 1). In the presence of 10 μM-$FeCl_3$, ascorbate oxidation was increased 4–5-fold by raising the pH from 6.5 to 10.0 (Fig. 1*a*). At pH 7.5 ascorbate oxidation was stimulated by $FeCl_3$ concentrations up to 10 μM, but higher $FeCl_3$ concentrations (up to 100 μM) produced no further stimulation (Fig. 1*b*). At pH 7.5, with 10 μM-$FeCl_3$, the addition of 100 μM-EDTA or -DTPA (1:1 ratios with ascorbate) decreased ascorbate oxidation rates to 8% and 2% (respectively) of the values reported in Fig. 1(*b*) (see

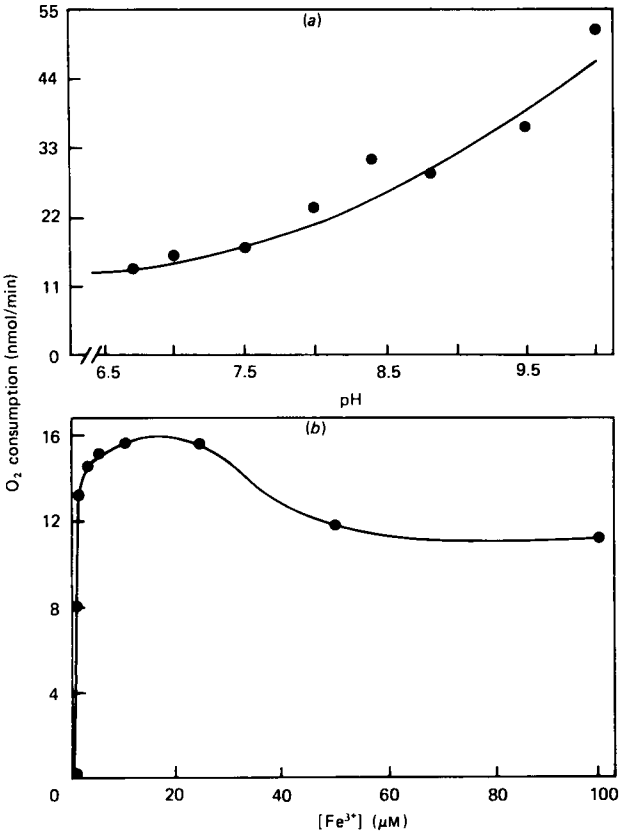

Fig. 1. Iron-ion- and pH-dependence of ascorbate oxidation

Initial rates of oxidation of 100 mM-ascorbate were measured by O_2 consumption at 25 °C, (*a*) with 10 μM-$FeCl_3$ at the pH values indicated, and (*b*) at pH 7.5 with the $FeCl_3$ concentrations indicated, in 100 mM-potassium phosphate buffer.

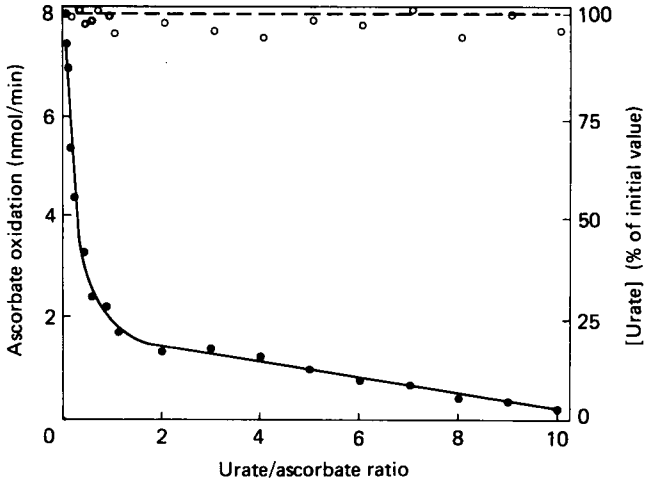

Fig. 2. Inhibition of ascorbate oxidation by urate

Initial rates of oxidation of 100 μM-ascorbate (●) measured by decrease in A_{265} in the presence of 10 μM-$FeCl_3$ ± urate, and percentages of initial urate concentrations (5.0 μM–1.0 mM) remaining (○) after 1 min incubations with 100 μM-ascorbate + 10 μM-$FeCl_3$. Experiments were conducted in 100 mM-potassium phosphate buffer, pH 7.5, at 25 °C.

below). At pH 12.0, no inhibitory effects of the iron-ion chelators EDTA and DTPA could be detected. These observations are in accord with previous findings [17] that copper-ion-catalysed ascorbate oxidation rates increase proportionally with the concentration of monoanionic ascorbate, whereas ascorbate oxidation in the absence of metal ion catalyst is proportional to the dianion concentration ($pK = 11.57$). It would thus appear that at physiological pH the existence of free iron (or copper) ions could have a significant effect on ascorbate oxidation. Spectrophotometric measurements of ascorbate oxidation were in complete agreement with the O_2-consumption measurements above (results not shown).

Urate inhibition of iron-ion-dependent ascorbate oxidation

In the presence of 10 μM-Fe^{3+} (pH 7.5), the oxidation of 100 μM-ascorbate was strongly inhibited by urate (Fig. 2). The degree to which urate inhibited ascorbate oxidation was a function of the urate/ascorbate ratio. At a urate/ascorbate ratio of 10:1, the inhibition of ascorbate oxidation was approx. 97%. The concentration-dependent inhibition by urate was found to be biphasic, with the greatest increase in inhibition occurring at urate/ascorbate ratios in the range 0–2:1 (82%). Increasing the urate/ascorbate ratio from 2:1 to 10:1 produced only a 15% further decrease in the rate of ascorbate oxidation. At a 1:1 urate/ascorbate ratio, ascorbate oxidation was decreased by approx. 72%. In comparison, 1:1 ratios of the iron-ion chelators EDTA and DTPA with ascorbate inhibited oxidation by approx.

Table 1. Inhibition of ascorbate oxidation at pH 7.5 and pH 12.0

Ascorbate oxidation was monitored spectrophotometrically as described in Fig. 2 legend. All inhibitors were used at 100 μM concentrations in the presence of 100 μM-ascorbate and 10 μM-$FeCl_3$ (at 25 °C). Values are means ± S.E.M. for five independent determinations. Abbreviation: N.S., no significant inhibition, $P > 0.05$ (*t* test).

	pH 7.5		pH 12.0	
Inhibitor	Rate (nmol of ascorbate/min)	Inhibition (%)	Rate (nmol of ascorbate/min)	Inhibition (%)
None	7.4 ± 0.4	—	56.3 ± 2.6	—
Urate	2.1 ± 0.1	72	55.9 ± 3.4	N.S.
EDTA	0.6 ± 0.1	92	57.2 ± 2.1	N.S.
DTPA	0.1 ± 0.1	98	54.8 ± 2.7	N.S.

92% and 98% respectively at pH 7.5 (Table 1). At pH 12.0, however, urate, EDTA and DTPA did not exhibit any detectable inhibition of ascorbate oxidation.

Spectrophotometric measurements also revealed that 100% of the urate added remained unoxidized after 1 min incubation with 100 μM-ascorbate + 10 μM-$FeCl_3$ (Fig. 2). Were uric acid to protect ascorbate by itself undergoing oxidation, one would expect to observe significant urate oxidation rates. It should also be remembered that initial rates of ascorbate oxidation are reported in Fig. 2, whereas urate was measured after 1 min incubation. With the spectrophotometric determination of uric acid, sensitivity is, naturally, greatest at low urate concentrations. In Fig. 2 urate concentrations of 5 μM and 10 μM provided approx. 7% and 28% protection respectively against ascorbate oxidation; although stoichiometric urate oxidation would certainly have been measurable under these conditions, none was observed. To check these results, the experiments of Fig. 2 were repeated, with the use of an h.p.l.c. method [18] to measure urate and ascorbate concentrations. The h.p.l.c. analysis completely confirmed the data of Fig. 2, including the observation that urate was not oxidized (results not shown). These results demonstrate that urate does not decrease the apparent rate of ascorbate oxidation by re-reducing oxidized ascorbate, since this would be expected to produce urate oxidation products, e.g. allantoin.

Although ascorbate oxidation by metal ions has been reported to be a stoichiometric event [17], we wanted to be sure that chain reactions were not occurring. If the oxidation of ascorbate by Fe^{3+} were to proceed via extended chain reactions of chain length 100, for example, only 1 μM-urate would need to be oxidized to provide complete protection by an antioxidant scavenger mechanism. Under even the best experimental conditions such low rates of urate oxidation would be difficult to measure accurately. We therefore determined the chain length of Fe^{3+}-catalysed ascorbate oxidation, using a method reported by Barclay *et al.* [19]. In this procedure the rate of ascorbate oxidation (in the presence and the absence of urate) was measured in the absence and in the presence of the radical-propagating species azobis-(2-amidinopropane hydrochloride). The rate of ascorbate (100 μM) oxidation was 7.4 ± 0.4 nmol/min, both in the absence and in the presence of 40 μM-azobis-(2-amidinopropane hydrochloride), and urate was an equally good inhibitor under both conditions. These results indicate that the oxidation of ascorbate by Fe^{3+} is, indeed, a stoichiometric reaction (i.e. a chain length of 1.0), and that urate does not inhibit ascorbate oxidation by a chain-terminating antioxidant scavenger mechanism.

It appeared possible that urate might inhibit ascorbate oxidation by reducing Fe^{3+} to Fe^{2+} (Fe^{2+} does not catalyse ascorbate oxidation). At pH 7.4, however, a 200 μM solution of urate reduced Fe^{3+} to Fe^{2+} at a rate of only 0.02 nmol/min (as measured by formation of *o*-phenanthroline–Fe^{2+} complex). This rate of Fe^{3+} reduction is much too low to explain the protection of ascorbate by urate. It should be noted, however, that urate is a fairly good reductant of Fe^{3+} at pH 3.0 (results not shown). It also appeared possible that urate might inhibit Fe^{3+}-dependent ascorbate oxidation by stabilizing Fe^{2+} in the reduced state. This possibility was ruled out by experiments in which the oxidation of 100 μM-$FeSO_4$ (in 100 mM-potassium phosphate buffer) was monitored at pH 7.4 at 25 °C. Under these conditions, the oxidation

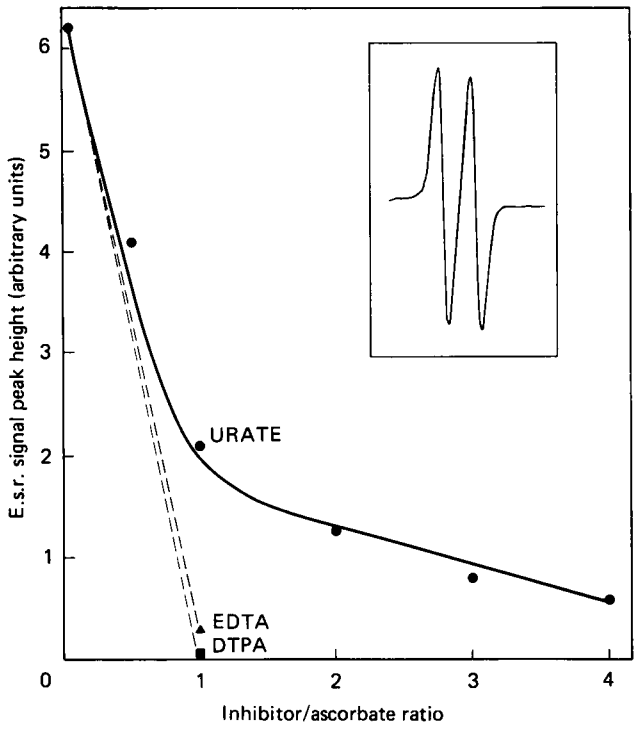

Fig. 3. Inhibition of ascorbyl radical production by urate

The ascorbyl radical ($g = 2.0052$) was studied by e.s.r. spectroscopy at 25 °C. Ascorbate, $FeCl_3$ and urate concentrations, as well as incubation conditions, were as reported in Fig. 2 legend except that ascorbate was introduced last by rapid mixing inside the e.s.r. cavity. EDTA (▲) and DTPA (■) were each used at a final concentration of 100 μM. All values are (upfield) maximal peak heights of the ascorbyl doublet spectrum (shown in the inset), which were achieved within 10 s of mixing. E.s.r. conditions: magnetic field, 334.0 mT (3340 G); microwave frequency, 9.44 GHz; modulation amplitude, 0.05 mT (0.5 G); time constant, 0.3 s; microwave power, 10 mW; scan range, 0.5×10.2 mT (0.5×102 G).

of Fe^{2+} to Fe^{3+} induced O_2 consumption (oxygen electrode) at a rate of 65 ± 3 nmol/min. In the presence of 2 mM-urate (other conditions unchanged) the rate of O_2 consumption by $FeSO_4$ was 71 ± 4 nmol/min. These results indicate that urate does not inhibit ascorbate oxidation by decreasing the rate of Fe^{2+} oxidation, or by maintaining Fe^{2+} in the reduced state.

It has been proposed (on theoretical grounds) that purines may form hydrogen bonds with ascorbate [20]. Since ascorbate oxidation occurs through abstraction of its enolic hydrogen atom by oxygen [21], hydrogen-bonding (with urate) should decrease both $FeCl_3$- and pH-dependent oxidation, but no protection at high pH was observed in the present study (Table 1). To explore this possibility further, we examined the proton-n.m.r. spectra of urate, ascorbate and urate + ascorbate mixtures. Although the n.m.r. method should be highly sensitive to hydrogen-bonding, no evidence for an ascorbate–urate complex was obtained. The lack of hydrogen-bonding between urate and ascorbate has recently been confirmed by Dr. Wolfgang Lohman (personal communication) of the University of Giessen, Giessen, West Germany, at both neutral and alkaline pH.

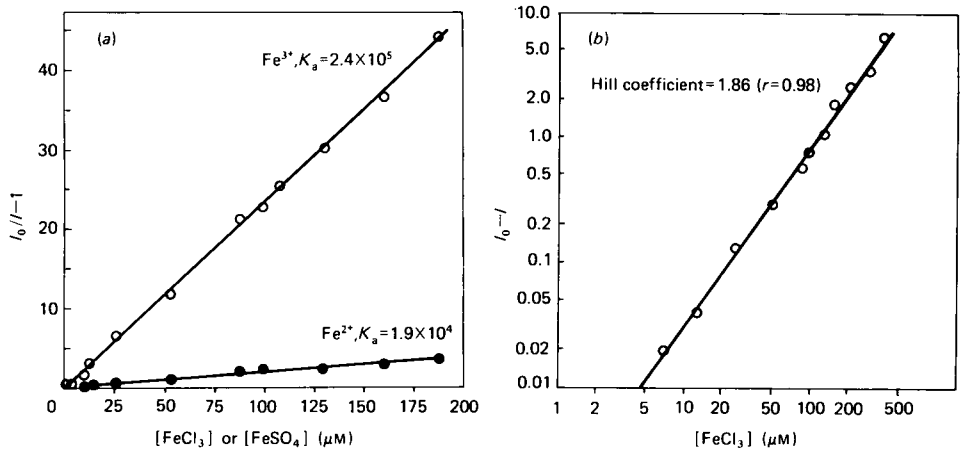

Fig. 4. Binding of iron ions by uric acid

(*a*) Fluorescence-quenching experiments to determine the association constants (K_a) for 1:1 urate complexes with Fe^{3+} ($FeCl_3$) and Fe^{2+} ($FeSO_4$). I_0 is the urate fluorescence in the absence of iron ions, and I is the urate fluorescence in the presence of various iron ion concentrations. Urate was used at a concentration of 10 μM for all data points. K_a was determined from the slope of the least-squares linear-regression curves for both Fe^{3+} ($r = 0.97$) and Fe^{2+} ($r = 0.98$). (*b*) Double-logarithmic plot of fluorescence quenching by Fe^{3+} ($FeCl_3$) in the presence of 0.5 mM-urate. The Hill coefficient was determined as the slope of the least-squares linear-regression curve ($r = 0.98$).

The oxidation of ascorbate occurs by two one-electron transfers, with the ascorbyl radical as the intermediary state. The rate-limiting step in the overall process is the first one-electron oxidation to the ascorbyl radical [21]. As shown in Fig. 3, production of ascorbyl radicals is greatly diminished by the addition of urate. The inhibition curve for Fig. 3 shows excellent agreement with that for Fig. 2, indicating that urate probably exerts its major effect by inhibiting the oxidation of dihydroascorbate to the ascorbyl radical (monohydroascorbate). This reasoning is further supported by the observation that rapid mixing of urate with an ascorbyl radical solution had little effect on the further oxidation of the ascorbyl radical to dehydroascorbate (results not shown). As might be expected, strong inhibition of (Fe^{3+}-dependent) ascorbyl radical production was provided by the iron-ion chelators EDTA (95%) and DTPA (98%) at 1:1 concentration ratios with ascorbate.

Since urate inhibits the iron-ion-dependent oxidation of dihydroascorbate to the ascorbyl radical without urate oxidation, Fe^{3+} reduction or ascorbate binding, we wondered if urate might form a stable complex with Fe^{3+}. This possibility is explored in the next section.

Formation of iron ion complexes by urate

Albert [22] has reported that urate and other purines have the ability to bind iron ions. Unfortunately, experimental difficulties have prevented the accurate determination of stability constants of urate–iron ion complexes. In our own studies we have found that potentiometric and spectrophotometric methods are not suitable for the study of urate–iron ion complexes owing to problems with solubility and pH-dependent oxidation/reduction reactions. We have therefore employed fluorescence-quenching techniques [14, 15] at pH 7.0, which do not require high concentrations of urate. The addition of $FeCl_3$ to uric acid caused a concentration-dependent loss of urate fluorescence, indicative of the formation of a non-fluorescent urate–iron ion complex; similar results were also obtained with $FeSO_4$. These experiments were conducted under conditions in which collisional quenching (transformation of electronic excitation energy into kinetic energy) was insignificant. The slope of a plot of $I_0/I-1$ versus iron ion concentration gives the association constant (K_a) for a 1:1 urate–iron ion complex (where I_0 is the urate fluorescence and I is the urate fluorescence in the presence of iron salt). As shown in Fig. 4, the K_a for a urate–Fe^{3+} (1:1) complex is 2.4×10^5, and the K_a for a urate–Fe^{2+} (1:1) complex is 1.9×10^4.

If urate could only form 1:1 complexes with iron ions, the K_a values determined above would also represent stability constants (K_s). Further experiments, however, revealed that the binding of the first urate molecule to iron ion facilitates the binding of a second urate molecule. In these experiments we measured the ability of relatively low (7–400 μM) concentrations of iron salts to quench the fluorescence of a more-concentrated urate solution (500 μM). Plots of I_0-I versus $FeCl_3$ concentration revealed increasing fluorescence quenching up to a urate/Fe^{3+} ratio of approx. 2:1 (i.e. 500 μM-urate/250 μM-$FeCl_3$), at which point maximal quenching was attained. Double-reciprocal plots revealed a clear sigmoidal relationship, indicative of 'co-operativity'. Double-logarithmic plots of these data were used to determine the Hill coefficient for the formation of urate–Fe^{3+} 2:1 complex (Fig. 4*b*). A Hill coefficient of 1.86 was determined from the slope of Fig. 4(*b*). An estimate of the K_a for the binding of the second urate molecule to Fe^{3+} (K'_a) may be obtained by multiplying the Hill coefficient (a measure of the increased affinity of the second urate molecule relative to the first) by the K_a for a 1:1 complex; thus $K'_a \simeq 1.86 \times (2.4 \times 10^5) \simeq 4.5 \times 10^5$. The overall stability constant (K_s) for urate–Fe^{3+} complexes may be estimated as the product of $K_a \times K'_a$; thus $K_s \simeq (2.4 \times 10^5) \times (4.5 \times 10^5) \simeq 1.1 \times 10^{11}$.

Binding to urate also caused a decrease in the redox potential of the Fe^{2+}–Fe^{3+} couple. The reduction potential for the Fe^{2+}/Fe^{3+} half-reaction was determined as −0.77 V (versus the normal hydrogen electrode), in

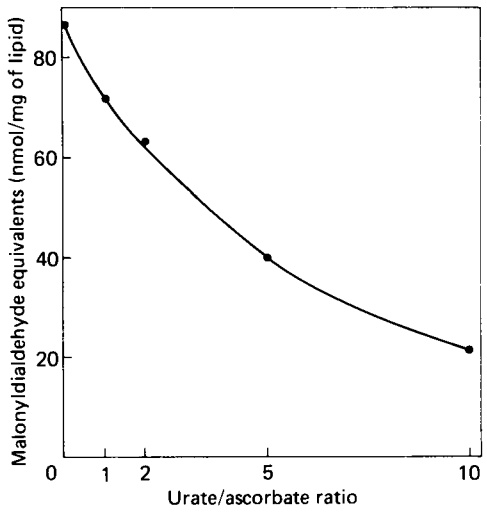

Fig. 5. Inhibition by urate of lipid peroxidation in liposomes

Malondialdehyde concentrations were measured by the thiobarbituric acid method (see the Materials and methods section) after 1 h incubations of unilamellar liposomes with ascorbate + $FeCl_3$ in the absence and in the presence of various concentrations of urate. Incubations were conducted in 100 mM-potassium phosphate buffer, pH 7.5, at 37 °C. Ascorbate concentration was 100 μM, and $FeCl_3$ concentration was 10 μM.

excellent agreement with established values [16]. Upon binding to urate, the Fe^{2+}/Fe^{3+} reduction potential decreased to −0.67 V. Thus the formation of urate–iron ion complexes decreases the Fe^{2+}/Fe^{3+} reduction potential by approx. 0.1 V. A urate–Fe^{3+} complex may therefore be a somewhat poorer oxidant for ascorbic acid than is $FeCl_3$.

Inhibition of ascorbate + iron-ion-dependent lipid peroxidation by urate

In the presence of 10 μM-$FeCl_3$ and 100 μM-ascorbate, the peroxidation of unsaturated fatty acids in unilamellar liposomes was readily observed (Fig. 5). The addition of urate decreased both the rate of ascorbate oxidation and the extent of lipid peroxidation, in a concentration-dependent manner. At an urate/ascorbate ratio of 10:1 (close to the situation in human blood) lipid peroxidation was inhibited by approx. 76% (Fig. 5). In comparison, ascorbate oxidation was inhibited by approx. 97% at a urate/ascorbate ratio of 10:1 (see Fig. 2). The inhibition curve for urate addition plotted versus malondialdehyde concentration (Fig. 5) was somewhat more shallow than the curve for urate addition plotted versus ascorbate oxidation (Fig. 2), with less inhibition occurring at low urate/ascorbate ratios. Nevertheless, the inhibitory effect of urate on liposomal lipid peroxidation was striking. The differences observed probably reflect interactions between Fe^{3+} and liposomal phospholipids, as well as dissimilarities inherent in comparisons of reaction rates and product (malondialdehyde) accumulation. This last point may be particularly important, since lipid-peroxidation initiation events are amplified by propagation reactions. In support of such interpretations, we can add the observation that EDTA was also a poorer inhibitor of liposomal lipid peroxidation than of ascorbate oxidation. When added at a 2:1 ratio with ascorbate + $FeCl_3$ (as per Fig. 5), EDTA inhibited lipid peroxidation (malondialdehyde) by 76%. In contrast, EDTA provided 97% protection against ascorbate oxidation (O_2 consumption) and 98% inhibition of ascorbyl radical production (e.s.r.) at a concentration ratio of 2:1 with ascorbate.

Table 2. Inhibition of microsomal lipid peroxidation by uric acid

Lipid peroxidation was measured by malondialdehyde determination (thiobarbituric acid assay) after incubation of liver microsomal fraction with 100 μM-ascorbate + $FeCl_3$ in the presence and in the absence of 500 μM-urate. Incubations were conducted at 37 °C for 15 min, as described in the Materials and methods section.

Concn. of $FeCl_3$ (μM)	Malondialdehyde formation (nmol/mg of protein)		Inhibition (%)
	Urate absent	Urate present	
1.0	0.93 ± 0.06	0.33 ± 0.04	64
10.0	1.33 ± 0.03	0.51 ± 0.06	62

Urate was also an effective inhibitor of lipid peroxidation in rat liver microsomal fraction (Table 2). The combination of ascorbate + $FeCl_3$ produced extensive lipid peroxidation, although neither agent was effective alone. Addition of urate (5:1 with ascorbate) inhibited lipid peroxidation by approx. 60–65% (Table 2). These results are in excellent agreement with the inhibition of lipid peroxidation exerted by urate in model liposomal systems above.

DISCUSSION

Urate has been shown to form stable co-ordination complexes with both Fe^{3+} and Fe^{2+}. For the formation of 1:1 complexes the K_a for Fe^{3+} is 2.4×10^5 and the K_a for Fe^{2+} is 1.9×10^4. Our data also indicate that urate can form a 2:1 complex with Fe^{3+}, in which the binding of the second urate molecule is favoured over the binding of the first urate molecule by a factor of approx. 1.86. From these relationships we can calculate that the association constant for the second urate molecule (K'_a) is approx. 4.5×10^5. A reasonable estimate of the overall stability constant (K_s) is provided by $K_a \times K'_a \simeq 1.1 \times 10^{11}$. Binding to urate caused a small decrease in the reduction potential of the Fe^{2+}/Fe^{3+} half-reaction from −0.77 V to −0.67 V, making urate–Fe^{3+} complexes somewhat poorer oxidants than free Fe^{3+}.

The overall reaction scheme for the oxidation of ascorbic acid by Fe^{3+} may be summarized by eqns. (1)–(4) below:

$$AH_2 + Fe^{3+} \rightarrow AH^{\cdot} + Fe^{2+} \quad (1)$$

$$Fe^{2+} + O_2 \rightarrow Fe^{3+} + O_2^{\cdot -} \quad (2)$$

$$AH^{\cdot} + O_2 \rightarrow A + O_2^{\cdot -} \quad (3)$$

Overall:

$$AH_2 + 2O_2 \rightarrow A + 2O_2^{\cdot -} \quad (4)$$

Eqns. (1) and (3) represent the first- and second-stage oxidations of ascorbic acid, where $AH^{\cdot}$ is the ascorbyl radical. At physiological pH, ascorbic acid ($pK_1 = 4.17$, $pK_2 = 11.57$) actually exists almost exclusively as the

ascorbate monoanion, AH^-. Eqn. (2) is the oxidation of Fe^{2+} produced in reaction (1), and eqn. (4) represents the overall stoichiometry of the two-step oxidation. It will be noted that eqns. (1)–(3) are rather simplistic reaction formulae that ignore possible intermediate complexes of ascorbate, iron ions and O_2. In particular, reaction (3) should be viewed as being conjectural, since evidence for ascorbyl radical reduction of O_2 is not available. In actuality, it has been shown that the catalytic effects of Fe^{3+} depend on complex-formation, as is also the case for copper-ion-catalysed ascorbate oxidation [23]. Ascorbate forms a relatively weak 1:1 monoprotonated chelate with Fe^{3+} that has a stability constant of approx. 10^2 [23].

Our results indicate that urate inhibits Fe^{3+}-dependent ascorbate oxidation by forming complexes with Fe^{3+}, thus blocking reaction (1), which is the rate-limiting step [21]. Several other possible explanations for the preservation of dihydroascorbate by urate have been ruled out. These include: (1) urate oxidation in a classical antioxidant scavenger reaction; (2) re-reduction of oxidized ascorbate by urate; (3) Fe^{3+} reduction to Fe^{2+} by urate (at pH 7.4); (4) inhibition of ascorbate radical chain reactions (ascorbate shows no evidence of chain oxidation); (5) inhibition of Fe^{2+} re-oxidation to Fe^{3+}; (6) inhibition of ascorbyl radical oxidation to dehydroascorbate; (7) formation of a stable urate–ascorbate hydrogen-bonded complex.

When we first proposed that urate could inhibit ascorbate oxidation and lipid peroxidation by forming co-ordination complexes with iron ions, we had only indirect evidence for urate–iron ion binding [25]. Shortly thereafter, Lam *et al.* [26] proposed that urate could inhibit copper-ion-dependent ascorbate oxidation by formation of stable urate–Cu^+ complexes. Unfortunately, Lam *et al.* were also unable to provide any direct evidence for the formation or stability of urate–copper ion complexes [26]. Smith & Lawing [27] had already shown that both urate and ribosylurate protected against copper-ion-induced peroxidation of arachidonate, linolenate and erythrocyte membrane lipids. By using e.s.r. techniques, we have recently found that urate also binds copper and manganese ions (K. J. A. Davies, unpublished work). It is therefore possible that the formation of urate–metal ion complexes may be a general mechanism whereby urate can inhibit the oxidation of ascorbate by transition-metal ions.

Recently we reported that uric acid appears to be responsible for the preservation of ascorbic acid in human blood [9]. Ascorbate oxidation in human serum showed evidence of dependence on endogenous transition-metal ions, and was inhibited by as much as 75% in the presence of physiological concentrations of urate; no urate oxidation was observed in these experiments [9]. The present results provide a clear rationale for the preservation of ascorbic acid in human blood, via scavenging of transition-metal ions by uric acid. Such thoughts take on greater significance when one considers that the evolutionary pressures which led to the loss of ascorbate synthetase activity in humans have also been accompanied by the loss of uricase activity and the development of efficient renal systems for the reabsorbtion of urate [2].

The effectiveness of ascorbate as a biological antioxidant may be limited by its propensity to oxidize, generating oxygen radicals and mutagens [24], and initiating lipid peroxidation [2]. Such properties represent the paradox of several common antioxidants, and may be viewed as a consequence of their somewhat delicate biological 'redox poise'. Ames *et al.* reported that uric acid is a highly effective scavenger in several lipid peroxidation systems, including γ-irradiation ($^{\cdot}OH$), photo-oxidation ($^1O_2$), haematin or haemoglobin + H_2O_2 (oxo-haem oxidants) and lipid hydroperoxides. Urate was also found to protect against the oxidative haemolysis of erythrocyte membranes by lipid hydroperoxides [2] or xanthine oxidase + acetaldehyde [2, 6]. A feature common to all systems in which urate inhibits peroxidation by quenching active species is that urate is oxidized to allantoin, and other products, in the process. In the present paper we have demonstrated an additional anti(per)oxidant activity of uric acid that is dependent upon its metal-ion-binding properties. As shown in eqn. (4) above, the oxidation of ascorbate produces two molecules of $O_2^{\cdot-}$. The $O_2^{\cdot-}$ so produced may then proceed, via iron-ion-catalysed Haber–Weiss reactions [28], Fenton reactions [29] or other (less-well-defined) chemistry [30], to initiate lipid peroxidation in liposomes and microsomal fraction (Fig. 5 and Table 2). The formation of urate–iron ion complexes prevents the initial generation of $O_2^{\cdot-}$ (since ascorbate is prevented from oxidizing) and so inhibits lipid peroxidation without causing urate oxidation. Although urate would also be expected to decrease lipid peroxidation in such systems by scavenging $^{\cdot}OH$ or other oxidants [2], our results indicate that the greater effect may be inhibition of the initial radical-producing step, iron-ion-catalysed ascorbate oxidation.

The effectiveness of urate as an inhibitor of ascorbate oxidation and lipid peroxidation in the present investigation, and in our previous work [9], was found to be a function of the urate/ascorbate concentration ratio. Concentrations of urate greatly in excess of available iron ions were used, which might imply an interaction between urate and ascorbate rather than between urate and iron ions. N.m.r. studies, however, revealed no such interaction between urate and ascorbate, and our results may be better explained on the basis of urate competition with ascorbate for available Fe^{3+}. On the basis of the approximate stability constants for Fe^{3+} complexes with urate (approx. 10^{11}) and ascorbate (approx. 10^2) one would predict that urate should be an effective inhibitor of ascorbate oxidation and lipid peroxidation at a 1:1 concentration ratio with ascorbate (although exact inhibition is difficult to predict, because of the non-limiting oxidation rate of the ascorbyl radical). In agreement with this prediction, a 72% inhibition of ascorbate oxidation was observed at a urate/ascorbate ratio of 1.0:1 (Fig. 2). Predictions of peroxidation inhibition would be extremely difficult, owing to the tremendous complications contributed by radical-propagation reactions.

The importance of transition-metal ions, such as iron and copper ions, in free-radical reactions is now widely recognized [28–30]. Debate continues over the physiological forms of catalytic transition-metal ions, but this is likely to be a protracted discussion. Intracellular and extracellular fluids contain many metalloproteins and metal-ion-binding/transport proteins and a host of small molecules that can form stable co-ordination complexes with metal ions. In the presence of multiple complex-forming agents, the distribution of transition-metal ions will be a function both of individual stability constants and the relative concentration of each agent. It is

important to note that a dynamic equilibrium must be reached in which each complex-forming agent will bind some of the available metal ion(s). Our estimate of 1.1×10^{11} for the overall stability constant of formation of the urate–Fe^{3+} complex, combined with the high concentration of urate normally found in human plasma, suggests that urate may be one of the major low-M_r iron-ion-binding agents in human blood. This hypothesis is given greater significance by our previous report that urate protects ascorbate against iron-ion-catalysed oxidation in human blood, without itself undergoing oxidation [9]. We propose that the newly demonstrated iron-ion-binding properties of uric acid, combined with its previously described scavenger functions [2], provide further evidence for the theory that urate plays a major role in the antioxidant defences of human beings.

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