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A study of the kinetics of the reduction of chromate by ascorbate under aerobic and anaerobic conditions

Paul O'Brien*† and Nesta Woodbridge

Department of Chemistry, Queen Mary and Westfield College, University of London, Mile End Road, London E1 4NS, U.K.

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Abstract—The reduction of chromate (2×10^{-4} mol dm^{-3}) by L-ascorbate ($(0.95–5.71) \times 10^{-3}$ mol dm^{-3}) has been studied by spectrophotometry under aerobic and anaerobic conditions. Phosphate, HEPES and Tris-HCl buffers were used in pH range 6.8–7.8 with $T = 25/26$°C, $I = 0.5/1.0$ mol dm^{-3}, $[O_2] = 0.03–7.17$ mg l^{-1}. The reaction was confirmed as first order with respect to both reactants. The rate determining step is suggested as formation of a chromate-ascorbate-ester. In phosphate and HEPES buffers the experimental, pseudo-first-order rate constants obtained in the absence of dioxygen were almost identical to those obtained in its presence. However in the presence of Tris-HCl buffer an oxygen dependence of the reaction was observed. This suggests that inhibition of the rate constant by dioxygen is a buffer dependent effect and is not an intrinsic feature of the redox reaction between chromate and ascorbate. The implications of these results for the mechanism of chromate toxicity are discussed. © 1997 Published by Elsevier Science Ltd.

Keywords: chromate; ascorbate; rate constants; dioxygen.

Chromium(VI) is an established human and animal carcinogen [1]. It is now well established that chromium(VI) alone does not cause DNA lesions *in vitro* [2,3] neither do the typical chromium(III) products of reduction [4] (however, some chromium(III) complexes can bind to DNA *in vitro* and this may be a route to toxicity). It has consequently been concluded that the species that causes oxidative DNA damage is generated during the intracellular reduction of chromium(VI) to chromium(III). Inside the cell chromate is believed to be reduced by enzyme systems in various organelles and by small molecules such as glutathione, ascorbic acid or cysteine. These molecules are known to generate reactive intermediates in the course of the reduction of chromate. Such intermediates can cause oxidative DNA damage such as strand breaks [5,6] and alkali labile (AP) sites [7], as well other lesions such as DNA-protein cross-links [8] and DNA inter-strand cross-links [9].

Chromium has an extremely complex redox chemistry in aqueous solutions. Many intermediates are formed which makes it difficult to understand the mechanism by which chromate reduction causes DNA damage. A likely candidate for involvement in DNA damage is Cr^{V}, its formation is a common feature of many *in vitro* and *in vivo* chromate reductions. Cr^{V} complexes are known to induce cleavage of DNA [6, 12]. Cr^{IV} [10] and Cr^{II} [11] and carbon based radical species [10] have also been identified and suggested as possible DNA damaging species. It is often suggested that hydroxyl radicals are involved but recent spin-trapping and competition kinetics studies [13] show that a simple mechanism involving these radicals is unlikely since the addition of the hydroxyl radical scavenger glucose has no protective effect on either the yields of radical adducts formed or the induction of single strand breaks caused by the reduction of chromate.

Many studies of oxidative DNA damage have concentrated on the chromate–glutathione reaction [6,14, 15]. Indeed until recently ascorbate had been rejected as playing a major role in the intercellular reduction of chromate. However, it has been shown that ascor-

*Author to whom correspondence should be addressed.

†Present address: Department of Chemistry, Imperial College of Science, Technology and Medicine, University of London, London SW7 2AY, U.K.

bate is stoichiometrically the most important reductant in many biological fluids (including lung fluid) [16,17].

The kinetics of the chromate–ascorbate reaction are of interest as mechanistic details of the reaction could provide an insight into which reactive intermediates are involved in the formation of strand breaks. The reduction has been extensively studied in acid medium [20,21] but until recently received little attention at neutral pH [22]. The accepted stoichiometry of the reaction is; 3 moles ascorbic acid reacts with 2 moles Cr^{VI}, each ascorbate transfers 2 electrons and overall 3 moles dehydroascorbic acid and 2 moles Cr^{III} are formed. No universally agreed stoichiometric equation has yet emerged, as the exact identity of the initial chromium and ascorbate species at physiological pH is not clear. Recently several kinetic studies of the chromate–ascorbate reaction have been published [23–25]. These confirm that the reaction is first order in both chromate and ascorbate. Several differing mechanisms were suggested, including the formation of a chromate-ascorbate ester or a Cr^{V}-ascorbate complex as the rate determining step. The reaction was found to be catalysed by copper(II), iron(II) and iron(III) ions [23] but was not inhibited by manganese(II) [24]. The reaction rate decreased as the ionic strength increased. However, none of these studies were carried out in buffers from which trace metals had been removed.

One current theory suggests that a direct interaction of a high valence oxo or molecular oxygen-chromium species capable of oxygen transfer with DNA plays an important role in the generation of oxidative DNA lesions [18,19,26]. Hence the kinetics of the reaction under both aerobic and anaerobic conditions are of interest. This paper reports the results of a kinetic study of the chromate–ascorbate reaction using UV techniques at low reactant concentrations in demetallated buffers, aerobic and anaerobic conditions were employed.

EXPERIMENTAL

Chemicals

L-Ascorbic acid, L-ascorbic acid-sodium salt, [4-(2-hydroxyethyl)-1-piperazine-ethane sulphonic acid] (HEPES), [tris(hydroxymethyl)methylamine-HCl (Tris-HCl), glucose oxidase (Type IIS from *Aspergillus niger*), catalase (from bovine liver) and chelex 100 resin were purchased from the Sigma chemical company. Sodium dichromate, β-D-glucose, sodium chloride, sodium perchlorate, disodium hydrogen orthophosphate-anhydrous, sodium dihydrogen orthophosphate and tris(hydroxymethyl)methylamine (Tris base) were purchased from BDH chemicals. Sodium chromate was obtained from Fisons. Nitrogen and argon were supplied by BOC.

All reagents used were analytical grade (except for the non-demetallated anaerobic reactions where general purpose reagents were used). Buffer solutions referred to as demetallated had any trace metals present removed by treatment with chelex 100 resin. Trace metal removal is considered complete when loss of absorbance of ascorbate at 265 nm is minimal (>0.5% in 15 min). The pH of the solutions was measured/checked using an Alpha 600 ion meter. Electronic spectra were measured using a Perkin-Elmer 330 spectrophotometer. The absorbance of the chromate ion at 370 nm ($\varepsilon = 4810\ M^{-1}\ cm^{-1}$) was followed.

Methods

For aerobic experiments [27] thermostatted stock solutions of sodium ascorbate, sodium chloride/sodium perchlorate and sodium dichromate were prepared immediately before use. The required volume of chloride/perchlorate and ascorbate stock solutions were pipetted into a 1 cm spectrophotometer quartz cell. A small amount of the chromate stock solution was injected into the thermostatted reaction cell using a microlitre pipette. The cell was stoppered, shaken and returned to the thermostatted sample compartment of the spectrophotometer. Continuous measurements of absorbance against time were made.

All stock solutions for the anaerobic study were flushed with oxygen-free nitrogen or argon (for between 20 min and 1 h). They were protected by rubber septa and handled using standard syringe techniques before being injected into a cell with a nitrogen or argon atmosphere. Oxygen was also removed using an enzymatic system. The ascorbate and chloride/perchlorate stock solutions were degassed for 15 min in a Decon FS 200 ultrasonic bath. A solution containing glucose oxidase, β-D-glucose and catalase was added to the stock solutions in the cell [28]. Nitrogen was then bubbled through the cell for 5 min before the dichromate stock solution was injected. The oxygen concentration in the nitrogen flushed solutions for each kinetic study was "estimated" by measurements with a Jenaway 9010 oxygen meter.

Pseudo-first-order rate constants were obtained graphically from plots of $\ln(A_{obs}/A_0)$ *vs* time. Plots were linear for 2–3 half lives (except for 0.95×10^{-3} mol dm^{-3} ascorbate experiments which were not under pseudo-first-order conditions and were only linear for 1–2 half lives). Reported values are the average of several runs, standard deviation is small, >5%.

RESULTS AND DISCUSSION

Aerobic conditions

The aerobic reduction of sodium dichromate by sodium L-ascorbate was studied under pseudo-first-order conditions at various values of pH. Under these conditions ascorbate gave a near first-order reduction

of chromate, however a second step towards the end of the reaction independent of ascorbate concentration was observed. The kinetics of the reaction under demetallated, aerobic conditions were not affected by either HEPES or phosphate buffer, reactions in either buffer gave near identical results in the pH range studied. Similarly perchlorate or chloride added for ionic strength gave near identical results. Rate constants were derived from plots of $\ln(A_{obs}/A_0)$ *vs* time. The observed pseudo-first-order rate constants were found to be directly proportional to the initial concentration of ascorbate as in previous non-demetallated studies [23–25]. The rate increased with ascorbate concentration (Fig. 1). Using the initial rates method a plot of log initial k_{obs} *vs* log ascorbate concentration proved to be linear with slope close to unity, confirming the reaction as first order with respect to ascorbate.

In this system chromium(VI) is present as either the hydrogen chromate ion or the chromate ion, at physiological pH and low Cr^{VI} concentrations other Cr^{VI} species are not significant [29]. Ascorbate is present as the strongly reducing hydrogen ascorbate ion [30]. Second order rate constants for the reaction can be calculated. The rate determining step of the reaction is probably the formation of a chromate-ascorbate-ester intermediate (Fig. 2). This step is thought to be slow compared to the subsequent reduction by pathways which are not yet clear but may involve Cr^{V},[31] Cr^{IV} [10] and perhaps Cr^{II} [11] before the final products of Cr^{III} and dehydroascorbic acid are formed. Cr^{VI} has a tendency to condensation reactions with many compounds [29] so the chromate-ascorbate-ester intermediate is likely, however due to the strongly reducing nature of the hydrogen ascorbate ion a rate determining step involving either a 1 : 1 reaction between a Cr species and ascorbate or electron transfer between HA^- and $HCrO_4^-$ cannot be ruled out. Assuming the rate of formation of the first step is slow compared to subsequent reactions then:

$$HA^- + HCrO_4^- \underset{k_b}{\overset{k_f}{\rightleftharpoons}} \text{products.} \qquad (1)$$

The resulting rate expression is:

$$k_{obs} = k_f[\text{ascorbate}]_0 + k_b. \qquad (2)$$

k_f and k_b can be directly ascertained graphically from plots of k_{obs} against $[\text{ascorbate}]_0$ (see Fig. 1 and Table 1). Since the forward reaction gives much larger values than the reverse reaction it can be assumed that k_b is insignificant as a reaction pathway. Estimates of the equilibrium constant K can be obtained since:

$$K = k_f/k_b. \qquad (3)$$

The pseudo-first-order rate constants were also found to be pH dependent, this was in agreement with previous non-demetallated results [23–25]. In the pH range 6.8–7.5 all ascorbate is present as HA^- [30] so the reactions present can be represented by:

$$HCrO_4^- \overset{K_a}{\rightleftharpoons} CrO_4^{2-} + H^+ (pK_a = 6.02), \qquad (4)$$

$$HCrO_4^- + HA^- \overset{k_1}{\rightarrow} \text{products}, \qquad (5)$$

$$CrO_4^{2-} + HA^- \overset{k_2}{\rightarrow} \text{products}. \qquad (6)$$

From these equations equation (7) can be arrived at:

$$k_{obs} = \frac{[H^+]k_1 + K_a k_2[\text{ascorbate}]_0}{[H^+] + K_a} + k_b. \qquad (7)$$

Since k_b is a minor reaction and $K_a \gg [H^+]$,

$$[HA]^- + [HCrO_4]^- \Leftrightarrow \text{A–Cr (VI)} + H_2O$$

Where A–Cr (VI) is:

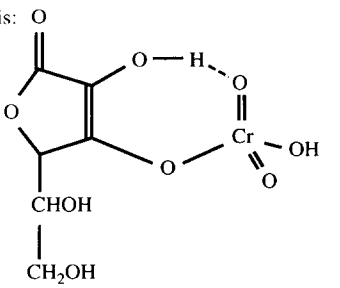

Fig. 2. Formation and structure of the chromate-ascorbate-ester.

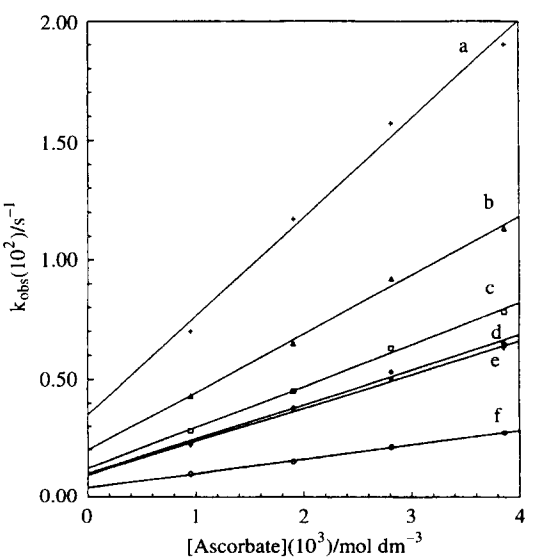

Fig. 1. Plot of k_{obs} *vs* $[\text{Na L-ascorbate}]_0$ for the reduction of chromate (2×10^{-4} mol dm^{-3}) by ascorbate at different pHs in demetallated HEPES/phosphate buffer (0.1 mol dm^{-3}, 25°C, $I = 1.0$). (a) pH 6.8; (b) pH 7.0; (c) pH 7.1; (d) pH 7.15; (e) pH 7.2; (f) pH 7.5.

Table 1. Second order kinetic parameters obtained from plotting k_{obs} *vs* [Na L-ascorbate]$_0$ for the aerobic reduction of chromate by ascorbate at different pH values. Using demetallated HEPES/phosphate buffer, 0.1 mol dm^{-3}, 25°C, $I = 1.0$

pH	k_f (dm^3 mol^{-1} s^{-1})	k_b (s^{-1} (10^3))	K (dm^3 mol^{-1} (10^3))
6.8	4.14(0.25)	3.50(0.63)	1.18
7.0	2.45(0.15)	2.00(0.31)	1.23
7.1	1.74(0.10)	1.21(0.20)	1.44
7.15	1.46(0.09)	1.00(0.15)	1.46
7.2	1.41(0.09)	0.95(0.20)	1.48
7.5	0.59(0.07)	0.42(0.15)	1.40

$$k_f = \frac{[H^+]k_1}{K_a} + k_2. \quad (8)$$

k_1 and k_2 can be ascertained graphically (from a plot of $[H^+]$ against k_f) as 32 ± 5.2 mol^{-1} dm^{-3} s^{-1} and -0.52 ± 0.25 mol^{-1} dm^{-3} s^{-1}, respectively (small negative figure for k_2 suggests it is insignificant).

The pH dependence could be explained if the formation of the chromate-ascorbate-ester is "promoted" by the presence of extra protons [22,29]. The slower reactions at higher pH values could be due to $^-$OH becoming the leaving group instead of water [22] (Fig. 3), however the small deviations from linearity of the plot of k_{obs} *vs* $[H^+]$ and the relatively large percentage error in k_1 and k_2 may suggest the role of H^+ is more complex than this.

Several detailed analyses of the kinetics of the chromate–ascorbate reaction have been undertaken [22–25], the only conclusions from all such studies and the present work is that the transient nature and large number of possible reaction intermediates involved make it very difficult to establish an overall mechanism for the process.

Anaerobic conditions

At the time our kinetic study was being carried out work was published which suggested that molecular oxygen was involved in the chromate–ascorbate reaction causing single strand breaks in PM2 DNA [18]. ESR evidence has suggested that molecular oxygen reacted with Cr^V to generate a formate cleaving reactive oxygen species [26]. We were interested in examining any oxygen dependence of the reaction kinetics to discover whether dioxygen was involved in a major mechanistic pathway. If it was, the observation would have important implications concerning the nature of the DNA cleaving species.

The anaerobic/partially anaerobic reduction of chromate by ascorbate was studied under pseudo-first-order conditions. Oxygen was partially removed by flushing with nitrogen or argon and also by using an enzyme system [28]. The reaction showed no oxygen dependence in demetallated HEPES or phosphate buffers at any pH (6.8–7.15) or ascorbate concentration ($(0.95–5.71) \times 10^{-3}$ mol dm^{-3}) at chromate concentration (2×10^{-4} mol dm^{-3}). Spectra taken from identical aerobic and anaerobic experiments were virtually identical (Fig. 4). No differences in the pseudo-first-order rate constants were seen. These results showed that the overall rate of reduction of chromate by ascorbate at physiological pH is not affected by the presence of molecular oxygen, which

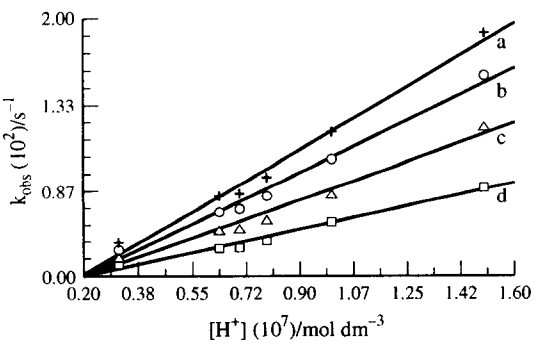

Fig. 3. Plot of k_{obs} *vs* $[H^+]$ for the reduction of chromate (2×10^{-4} mol dm^{-3}) by ascorbate at different [Na L-ascorbate]$_0$ in demetallated HEPES/phosphate buffer (0.1 mol dm^{-3}, 25°C, $I = 1.0$). (a) 0.95×10^{-3} mol dm^{-3}; (b) 1.90×10^{-3} mol dm^{-3}; (c) 2.86×10^{-3} mol dm^{-3}; (d) 3.81×10^{-3} mol dm^{-3}.

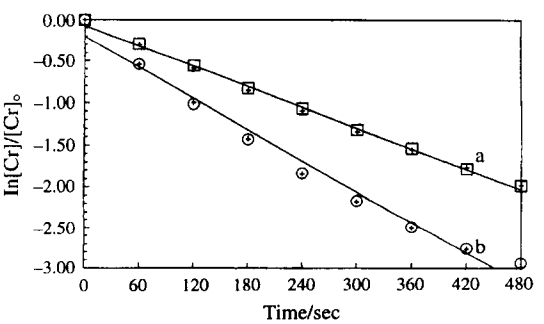

Fig. 4. An example of the lack of oxygen dependence of the reduction of chromate (2×10^{-4} mol dm^{-3}) by ascorbate in demetallated HEPES/phosphate buffer (pH 7.1, 0.1 mol dm^{-3}, 25 C, $I = 1.0$) (a) 1.90×10^{-3} mol dm^{-3}; (b) 3.81×10^{-3} mol dm^{-3}. A pseudo-first-order plot of $\ln[CrO_4^{2-}]/[CrO_4^{2-}]_0$ *vs* time. + Aerobic reactions, □ partially anaerobic reactions (40 min N_2/Ar), ○ anaerobic reactions (enzymes).

suggests that if oxygen is involved in the mechanism by which DNA cleavage occurs then the oxidising species is not formed in a major stoichiometric path of the chromate–ascorbate reduction.

However, as the studies in our laboratory were underway, Dixon *et al.* [25] reported a kinetic study in non-demetallated Tris-HCl buffer showed an up to a ten-fold increase in the reaction rate for experiments carried out in the absence of dioxygen. These results were in direct contrast to those established in the present study. We reexamined our results by undertaking studies to ascertain whether the retardation of reaction rate by dioxygen was due to an interaction between oxygen and a reactive intermediate formed in the chromate–ascorbate reaction which would deplete the amount of the reactive intermediate available for involvement in the rate-determining step. Alternatively the inhibition of the reaction kinetics by dioxygen could be caused by the choice of buffer or by the presence of trace metal ions perhaps activated by the buffer.

There are problems associated with trace metal ion contamination in these redox systems. The kinetics of ascorbate decomposition reactions are particularly sensitive to trace metals such as Fe^{II} and Cu^{II} [32]. Removing trace metal ions ensures that the observed kinetics are due to the reduction of the chromate ion alone. Tris-HCl buffer also has problems associated with its use. It is known to affect reductions [10,31], presumably by coordinating to Cr^{V} to form a ternary Cr^{V}/ascorbate/tris complex or a Cr^{V}/tris complex when used as a buffer for the chromate-ascorbate reaction. Indeed there were considerable problems in observing Cr^{V} in early work on chromate–glutathione reactions due to choice of Tris-HCl as a buffer [22]. Due to its participation in the Cr^{VI}/Cr^{V} system it is now rarely used for buffering chromate reactions.

In order to understand better the discrepancies between our results and those published [25], the reduction of chromate by L-ascorbic acid was studied at different stages of deoxygenation at a set pH and concentrations of ascorbate and chromate similar to those used by Dixon *et al.* [25] in several different non-demetallated buffers. Under these conditions all experiments gave first order reduction of chromate (Fig. 5). The large differences in rate constants between the buffers under aerobic conditions are probably partially due to the presence of differing amounts of trace metals. Phosphate is known to have high metal complexing ability [33]. In non-demetallated phosphate buffer the observed rate of the chromate reduction was not first order, the reaction actually had a mixed order with respect to the oxidant due to the large contribution from associated metal ions (mainly from copper(II)). A first order rate constant was estimated from the initial 4 min of the reaction. Tris-HCl buffer probably had more associated metal ions than HEPES buffer but both gave first-order rate constants. Since the reaction rate in phosphate buffer was not affected by the oxygen concentration (Table 2) it is likely that the reported oxygen dependence was not caused by trace metals alone. In HEPES and phosphate buffers the concentration of oxygen present in the reaction system had no effect on the kinetics of the chromate–ascorbate reaction. However in Tris-HCl buffer the first-order rate constants for the reduction of chromate by ascorbate increased in a non-linear fashion as oxygen concentration decreased. These results could not be repeated for demetallated experiments, perhaps because the reaction system was extremely sensitive to the amount of oxygen present, (injecting even small amounts of air into the reaction cell led to no increase in reaction rate being observed) maybe because the oxygen dependence is a buffer/trace metal ion effect. This experiment showed that the reported oxygen dependence of the chromate–ascorbate reaction was caused by the use of non-demetallated Tris-HCl buffer and was not an intrinsic feature of the redox reaction between chromium(VI) and ascorbate. This conclusion is supported by a recent ESR study [10] which showed that for the chromate–ascorbate reaction in demetallated HEPES buffer neither Cr^{V} or the for-

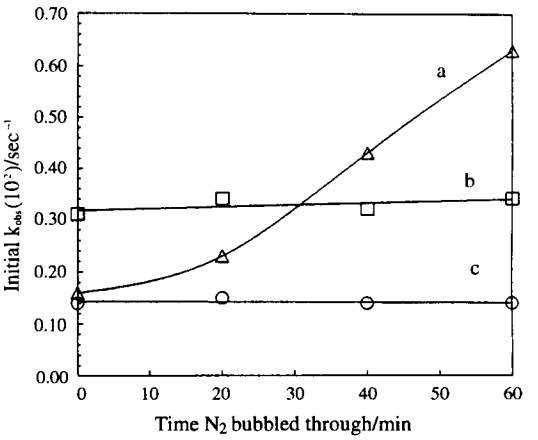

Fig. 5. Effect of dioxygen variation. Plot of k_{obs} *vs* length of time reaction solutions were flushed with nitrogen. Sodium chromate (2×10^{-4} mol dm^{-3}) was reduced by L-ascorbic acid (1×10^{-3} mol dm^{-3}) in non-demetallated buffers (pH 7.8, 0.05 mol dm^{-3}, 26°C, $I = 0.5$ ($NaClO_4$). (a) Tris-HCl buffer; (b) phosphate buffer; (c) HEPES buffer.

Table 2. [O_2] was estimated from solutions of the reaction mixture using a Jenway 9010 oxygen meter which was only accurate to about 0.05 mg l^{-1}. The oxygen concentration for the aerobic solution was very close to the literature value for solution with the salinity of the reaction mixture [34]

N_2 flushed (min)	Estimated [O_2] (mg l^{-1})
0	7.17
20	0.11
40	0.05
60	0.03

mation of carbon-based radicals was affected by the absence of dioxygen.

Our conclusion is not that dioxygen and reactive oxygen species do not have a role in DNA cleavage by the chromate-ascorbate system, just that the species involved in a major mechanistic pathway cannot be involved in DNA cleavage if oxygen is proved to be involved. An interaction between dioxygen and a chromium reaction intermediate involving Tris-HCl buffer (probably with Fe^{II} or Cu^{II} also involved) is a likely cause of the reported oxygen dependence of the chromate–ascorbate reduction.

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