Biochemical Pharmacology, Vol. 38, No. 11, pp. 1779–1786, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

SYNERGISTIC INTERACTIONS BETWEEN NADPH–CYTOCHROME P-450 REDUCTASE, PARAQUAT, AND IRON IN THE GENERATION OF ACTIVE OXYGEN RADICALS

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(*Received* 23 *May* 1988; *accepted* 3 *October* 1988)

Abstract—The toxicity associated with paraquat is believed to involve the generation of active oxygen radicals and the production of oxidative stress. Paraquat can be reduced by NADPH–cytochrome P-450 reductase to the paraquat radical; this results in consumption of NADPH. A variety of ferric complexes, including ferric–ATP, –citrate, –EDTA, ferric diethylenetriamine pentaacetic acid and ferric ammonium sulfate, produced a synergistic increase in the paraquat-mediated oxidation of NADPH. This synergism could be observed with very low concentrations of iron, e.g. 0.25 μM ferric–ATP. Very low rates of hydroxyl radical were generated by the reductase with paraquat alone, or with ferric–citrate or –ATP or ferric ammonium sulfate in the absence of paraquat; however, synergistic increases in the rate of hydroxyl radical generation occurred when these ferric complexes were added together with paraquat. Ferric–EDTA and –DTPA catalyzed some production of hydroxyl radicals, which was also synergistically elevated in the presence of paraquat. Ferric desferrioxamine was essentially inert in the absence or presence of paraquat. This enhancement of hydroxyl radical generation was sensitive to catalase and competitive scavengers but not to superoxide dismutase. The interaction of paraquat with NADPH–cytochrome P-450 reductase and ferric complexes resulted in an increase in oxygen radical generation, and various ferric complexes increased the catalytic effectiveness and potentiated significantly the toxicity of paraquat via this synergistic increase in oxygen radical generation by the reductase.

The toxicity associated with paraquat appears to involve events related to oxidative stress [1–5] and is believed to be due to the one electron reduction of paraquat to paraquat radical as catalyzed by various flavoprotein reductases [2, 3, 6, 7]. In the presence of oxygen, the paraquat radical rapidly auto-oxidizes with a rate constant of $7.7 \times 10^8\ M^{-1}\ sec^{-1}$ [8] to produce superoxide and, subsequently, H_2O_2 and other potent oxidants [4, 5, 9–15]. While earlier reports suggested the possibility that paraquat toxicity may occur independent of a role for transition metals, many recent reports have indicated an essential role for metals such as iron or copper in promoting paraquat toxicity [9, 11–13, 15–19]. This essential role for iron in promoting xenobiotic toxicity has been extended to other redox cycling toxic agents such as adriamycin® (doxorubicin hydrochloride)† [20–22], mitomycin C [23] and acetaminophen [24] although the exact mechanism for the role of the iron is not clear.

The addition of paraquat to rat liver microsomes was shown recently to result in an increase in microsomal generation of ·OH or ·OH-like species as reflected by the generation of ethylene from KMBA‡ or of acetaldehyde from ethanol [25]. This increase in microsomal ·OH generation is sensitive to catalase but not to superoxide dismutase. The increase can be observed in the absence of *added* iron (although as isolated, microsomes contain small amounts of non-heme iron), and there is a potentiation of the paraquat-mediated increase in the presence of certain ferric complexes, such as ferric–EDTA, but not others, such as ferric–desferrioxamine [25]. Since paraquat is reduced to paraquat radical via the NADPH–cytochrome P-450 reductase, and in order to lessen the complexities associated with iron in the microsomes and the direct oxidation of certain ·OH scavengers such as ethanol by cytochrome P-450 itself [26–28], the current studies were carried out to extend the previous work with microsomes to a more well-defined system involving the reductase itself, and to other ferric chelates. This system also allows a direct comparison of the ability of paraquat plus different ferric chelates to potentiate NADPH oxidation by the reductase to their ability to synergistically increase ·OH production. The ferric complexes which were utilized included ferric–EDTA and –DTPA, which have been shown to be very effective in catalyzing microsomal ·OH generation and in serving as electron acceptors from the reductase [29, 30], ferric desferrioxamine, which blocks ·OH generation by various systems, including the microsomes [31], and ferric–ATP or ferric–citrate, which may reflect more physiologically-relevant iron complexes.

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† Adriamycin is a registered trademark of Farmitalia Carlo Erba.

‡ Abbreviations: DTPA, diethylenetriamine pentaacetic acid; DFOA, desferrioxamine; FMN, flavin mononucleotide; KMBA, 2-keto-4-thiomethylbutyric acid; PMSF, phenylmethylsulfonylfluoride; and SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis.

MATERIALS AND METHODS

NADPH–Cytochrome P-450 reductase was puri-

fied by a slight modification of the method of Guengerich and Martin [32] from livers of male Sprague–Dawley rats. The minor changes included the use of 0.1% Emulgen 911 instead of 0.1% Lubrol, the omission of PMSF, and the use of 5 μM, rather than 2 μM, FMN. Final preparations were homogeneous on SDS–PAGE and had specific activities ranging from 36 to 48 units/mg protein. One unit of reductase activity is defined as an initial rate of 1 micromole of cytochrome *c* reduced per minute at 22° when assayed as described by Phillips and Langdon [33].

The oxidation of NADPH was determined by following the change in absorbance at 340 nm at room temperature of a reaction mixture containing 50 mM potassium phosphate buffer, pH 7.6, 0.1 mM NADPH, and 0.16 to 0.32 units of the reductase in a final volume of 1 ml. The production of 'OH or 'OH-like species was evaluated by following the production of acetaldehyde from ethanol or of ethylene from KMBA. The reaction mixture contained 50 mM potassium phosphate buffer, pH 7.6, 0.4 mM NADPH, 0.32 units of reductase, and either 1 mM KMBA or 50 mM ethanol as the 'OH scavenger substrate in a final volume of 1 ml. Experiments were conducted at 37° in 12 ml tubes capped with rubber serum stoppers and, at the indicated time periods, 1 ml of the head space was removed with a gas-tight syringe and injected into a Hewlett–Packard model 5750 gas chromatograph, and the production of acetaldehyde or of ethylene was determined as previously described [34, 35]. The production of H_2O_2 was determined by measuring the formation of acetaldehyde from the oxidation of ethanol by the catalase–compound I complex [36]. The reaction mixture contained 50 mM potassium phosphate, pH 7.6, 0.4 mM NADPH, 0.33 units of reductase, 100 mM ethanol, and 1150 units of catalase in a final volume of 1 ml. Reactions were initiated by the addition of reductase and were carried out at 37° in 12 ml tubes capped with serum stoppers. At appropriate time points, 1 ml of the head space was removed and the concentration of acetaldehyde was determined as described above.

All reagents were of the highest grade available. The phosphate buffer and the water used to prepare all solutions were passed through resins containing Chelex-100 to remove metal contaminants. NADPH, paraquat, EDTA, DTPA, KMBA, ATP and citrate were from the Sigma Chemical Co. (St Louis, MO); desferrioxamine was from Ciba Geigy (Desferral); ferric-ammonium sulfate and most other inorganic salts were from Fisher Scientific Co. (Fairlawn, NJ). The ferric complexes were prepared fresh by dissolving ferric ammonium sulfate in HCl and then diluting with the respective chelator to the appropriate stock concentrations. Ferric–ATP was utilized as a 1:20 complex, whereas the other ferric chelates were all utilized as a 1:2 complex.

RESULTS

Effect of paraquat and ferric chelates on NADPH oxidation. Since the initial event in the generation of free radicals by the reductase must involve the oxidation of NADPH, the possible synergistic interactions between paraquat and ferric chelates on NADPH oxidation by the reductase were investigated. In the absence of an electron acceptor, very low or essentially zero rates of NADPH oxidation by the NADPH–cytochrome P-450 reductase were observed. Certain ferric complexes such as ferric–ATP or –citrate or unchelated ferric ion (probably to a large extent ferric phosphate) were shown previously to be poor electron acceptors from the reductase [29, 30]. As shown in Table 1, these ferric complexes minimally promoted NADPH oxidation. Paraquat (0.1 mM) did serve as an effective electron acceptor from the reductase and promoted NADPH oxidation (Table 1). In the presence of ferric ammonium sulfate, ferric–ATP or –citrate, there was an increase in the ability of paraquat to promote the oxidation of NADPH by the reductase.

In contrast to the above ferric complexes, ferric–EDTA and ferric–DTPA chelates can serve as effective electron acceptors from the reductase [29, 30] and, as shown in Table 1, these ferric chelates did promote oxidation of NADPH by the reductase. The combination of paraquat plus ferric–EDTA or ferric–

Table 1. Effect of paraquat and ferric complexes on oxidation of NADPH by NADPH–cytochrome P-450 reductase

Ferric complex	Rate of NADPH oxidation (nmol/min)		Observed rate / Additive rate
	− Paraquat	+ Paraquat	
None	0	22	—
Ferric–NH_4SO_4	0	88	4.0
Ferric–Citrate	2	48	2.0
Ferric–ATP	0	77	3.5
Ferric–DTPA	22	200	4.5
Ferric–EDTA	17	85	2.2
Ferric–Desferrioxamine	3	29	1.3

The oxidation of 0.1 mM NADPH by 0.32 units of reductase was assayed as described in Materials and Methods. Final concentrations of iron and paraquat were 25 or 100 μM respectively. The results in column 4 were calculated from the rates in column 3 divided by the sum of the rate with paraquat in the absence of added iron (22 nmol/min) plus the rates in column 2 for each ferric complex.

Table 2. Concentration curves for the stimulation of NADPH oxidation by paraquat and ferric–ATP

	Concentration of paraquat (μM)	Concentration of ferric–ATP (μM)	Rate of NADPH oxidation (nmol/min)	Percent increase by ferric–ATP (%)
(A)*	50	0	11.2	
	50	0.25	17.1	53
	50	2.5	20.9	87
	50	5.0	21.6	93
	50	25.0	25.3	126
	50	50.0	25.4	127
(B)†	0	25.0	0	
	1	0	0.9	
	1	25.0	2.6	189
	5	0	2.4	
	5	25.0	5.7	138
	50	0	8.4	
	50	25.0	21.7	158
	100	0	20.2	
	100	25.0	37.5	86

* Experiment A: The oxidation of 0.1 mM NADPH by 0.16 units of NADPH–cytochrome P-450 reductase was assayed in the presence of 50 μM paraquat plus the indicated concentrations of ferric–ATP.

† Experiment B: The concentration of paraquat was varied over the range of 1 to 100 μM in the absence or presence of 25 μM ferric–ATP.

DTPA resulted in an increase in the oxidation of NADPH. For comparative purposes, the effect of ferric–desferrioxamine was examined since this iron complex is essentially inert in promoting microsomal lipid peroxidation or ·OH production [30, 31]. Ferric–desferrioxamine was a very weak electron acceptor from the reductase in the absence of paraquat; a small synergistic interaction between reductase, paraquat and ferric desferrioxamine was observed (Table 1). With all the ferric complexes evaluated, the rate (Table 1) as well as the extent (data not shown) of NADPH oxidation was increased in the presence of paraquat (as compared to values found for paraquat alone). These increases were greater than those expected from the sum of the values found for paraquat alone plus ferric chelate alone (column 4, Table 1), i.e. there was synergistic interactions between paraquat and the ferric complexes in promoting the oxidation of NADPH by the reductase.

In view of its wide use and possible physiological relevance, some further characterization of the ferric–ATP plus paraquat system was conducted. When the concentration of paraquat was kept constant at 50 μM, increasing the amount of ferric–ATP over the range of 0.25 to 50 μM produced an increase in the rate and extent of NADPH oxidation (Table 2). The interaction with paraquat appeared to be maximal at an iron concentration of about 25 μM, and a synergistic increase in NADPH oxidation by the reductase could be found with as little as 0.25 μM ferric–ATP. Dose–response studies indicated that in the presence of 25 μM ferric–500 μM ATP, a synergistic increase in NADPH oxidation could be found with 1 μM paraquat, which became more notable as the concentration of paraquat was elevated (Table 2).

The increase in the rate of NADPH oxidation by the reductase in the presence of paraquat or paraquat plus 25 μM ferric–ATP was identical in the absence or presence of 0.3 units superoxide dismutase or 230 units of catalase (data not shown). There was less than 10% inhibition of the rate of NADPH oxidation by 50 mM dimethyl sulfoxide (DMSO) or 100 mM ethanol in the presence of paraquat plus ferric–ATP, indicating little significant oxidation of NADPH by ·OH at least over this 2-min time course.

Hydroxyl radical production by reductase. The generation of ·OH or potent oxidants with the oxidizing power of ·OH was evaluated by assaying for the production of acetaldehyde from ethanol or of ethylene from KMBA. Table 3 shows that incubating the reductase with NADPH plus paraquat resulted in a small, time-dependent, production of acetaldehyde. As described previously [29], ferric–EDTA but not ferric–ATP served as an effective catalyst for ·OH generation by the reductase. The combination of paraquat plus ferric–EDTA resulted in a 4-fold greater rate of acetaldehyde generation than the sum of their individual rates (Table 3, 5-min time point). A striking synergistic interaction between paraquat plus ferric–ATP in stimulating ·OH generation by the reductase was observed at all time points (Table 3).

More detailed studies were conducted with KMBA as the ·OH scavenger in view of its increased sensitivity, which allowed more accurate detection of low rates of ·OH generation. Paraquat, in the absence of added iron, increased ethylene generation (Table 4), analogous to results with acetaldehyde production from ethanol. Ferric ammonium sulfate, ferric–ATP and ferric–citrate were not effective as catalysts of ethylene production in the absence of paraquat, but became effective catalysts in the pres-

Table 3. Synergistic interactions between reductase, paraquat and iron in oxidation of ethanol

Addition	Acetaldehyde production (nmol) Time (min) 5	10	15
Paraquat	0.9	2.2	2.8
Ferric–ATP	0	0	2.0
Paraquat + Ferric–ATP	24.5	35.1	42.7
Ferric–EDTA	8.9	23.8	35.6
Paraquat + Ferric–EDTA	44.2	59.9	67.0

The reaction mixture contained 50 mM potassium phosphate, pH 7.6, 50 mM ethanol and 0.32 units of reductase in a final volume of 1 ml. When present, the final concentration of paraquat was 0.2 mM, while the ferric complexes were added to a final concentration of 0.1 mM. Reactions were initiated by the addition of 0.4 mM NADPH, and acetaldehyde generation was determined by a head space gas chromatography procedure.

Table 4. Synergistic interactions between reductase, paraquat and various ferric complexes in the generation of hydroxyl radical

Ferric complex	Rate of ethylene production (nmol/min) − Paraquat	+ Paraquat	Observed rate Additive rate
None	0	0.019	—
Ferric–NH_4SO_4	0	0.084	4.4
Ferric–Citrate	0.007	0.169	6.5
Ferric–ATP	0.004	0.160	7.0
Ferric–DTPA	0.040	0.327	5.5
Ferric–EDTA	0.117	0.499	3.7
Ferric–Desferrioxamine	0	0.034	1.8

The oxidation of 1 mM KMBA to ethylene by 0.32 units of NADPH–cytochrome P-450 reductase was assayed as described in Materials and Methods. Reactions were carried out over a 15-min time period, and rates were calculated over the linear time course. All ferric complexes were present at a final concentration of 25 μM. Paraquat, when present, was added at a final concentration of 0.1 mM. The results in column 4 were calculated from the rates in column 3 divided by the sum of the rate with paraquat in the absence of added iron (0.019 nmol/min) plus the rates in column 2 for each ferric complex.

ence of paraquat (Table 4). The last column of Table 4 shows the ratio of ethylene production observed in the presence of paraquat plus the ferric complex divided by the sum of the rates produced by paraquat alone plus the ferric complex alone. Synergistic interactions between paraquat and ferric–ATP or –citrate, and to a lesser extent non-complexed ferric ion (ferric–phosphate), can readily be observed.

Similar experiments were conducted with ferric–EDTA, –DTPA, and –desferrioxamine chelates (Table 4). The latter was essentially inert in catalyzing the generation of $^{\cdot}OH$, even in the presence of paraquat. Ferric–EDTA and to a lesser extent ferric–DTPA were effective catalysts of $^{\cdot}OH$ production in the absence of paraquat, and their catalytic effectiveness increased in the presence of paraquat; clear synergistic interactions between paraquat and these two ferric chelates were observed (last column, Table 4).

The ability of ferric–ATP to synergistically interact with paraquat to catalyze the production of ethylene was dependent on the concentration of ferric–ATP. A synergistic interaction could be observed at a concentration of 2.5 μM ferric–50 μM ATP, with increasing effectiveness as the concentration of ferric–ATP was elevated (Fig. 1).

Effect of radical scavengers on KMBA oxidation by reductase. The synergistic interaction between paraquat and ferric–ATP in catalyzing the oxidation of KMBA to ethylene was insensitive to superoxide dismutase (up to 0.5 units/ml). However, catalase was a potent inhibitor of KMBA oxidation under all reaction conditions; the elevated rate of ethylene generation found in the presence of paraquat plus ferric–ATP was decreased about 80% by catalase (1150 units/ml) (data not shown), implicating a role for H_2O_2 as the precursor of the oxidant responsible for KMBA oxidation. To further implicate a role of $^{\cdot}OH$-like species in the increased oxidation of KMBA produced in the presence of paraquat plus ferric–ATP, the effect of competitive $^{\cdot}OH$ scavenging agents was assessed. Ethanol, DMSO and

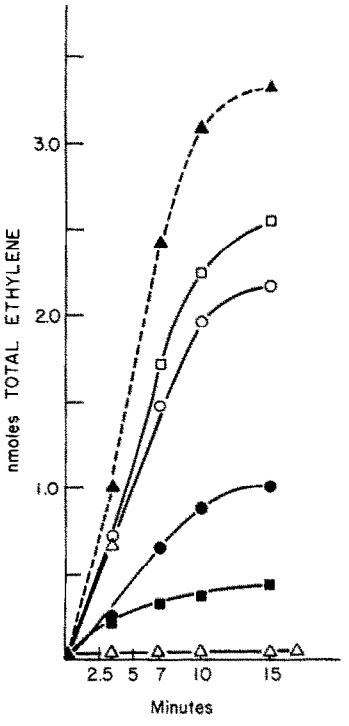

Fig. 1. Concentration curve for the stimulation of ethylene generation from KMBA by ferric–ATP. The oxidation of KMBA by 0.32 units of reductase was assayed in the presence of 0.1 mM paraquat alone (■—■) or 0.1 mM ferric–ATP alone (△—△) or 0.1 mM paraquat plus either 2.5 μM (●—●), 25 μM (○—○), 50 μM (□—□) or 100 μM (▲—▲) ferric–ATP respectively.

mannitol produced inhibition of ethylene generation (Fig. 2); the order of inhibitory effectiveness (DMSO > ethanol > mannitol) is in the same direction for the rate constants for these scavengers for reaction with ·OH [37].*

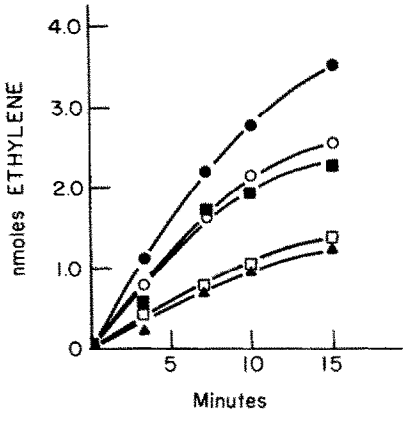

Fig. 2. Inhibition of ethylene production by competitive ·OH scavenging agents. The oxidation of KMBA to ethylene was determined in a reaction system containing 0.32 units of reductase, 0.1 mM paraquat and 25 μM ferric–ATP. Key: (●—●) control; (○—○) 50 mM mannitol; (■—■) 10 mM ethanol; (□—□) 100 mM ethanol; and (▲—▲) 50 mM dimethylsulfoxide.

H_2O_2 production by the reductase. In view of its critical role as the precursor of ·OH, the production of H_2O_2 by the reductase was determined. In the absence of paraquat or iron complexes, no H_2O_2 could be detected (data not shown), consistent with the very low rate of NADPH oxidation. Paraquat itself produced a striking increase in the rate of H_2O_2 generation (Fig. 3). Ferric–EDTA and, to a much lesser extent, ferric–ATP also produced an increase in H_2O_2 production, whereas ferric–desferrioxamine did not. There was no increase in the rate or amount of H_2O_2 produced in the presence of paraquat plus either ferric–EDTA or ferric–ATP over that produced by paraquat itself (Fig. 3). A possible explanation for this may involve the increasing utilization of H_2O_2 to synergistically produce ·OH in the presence of paraquat plus either ferric–EDTA or ferric–ATP. In fact, H_2O_2 production was decreased slightly in the presence of ferric–EDTA or –ATP plus paraquat compared to that found with paraquat alone. H_2O_2 generation was identical for systems containing paraquat or paraquat plus ferric–desferrioxamine (Fig. 3) since there was no synergism in ·OH production by paraquat when ferric–desferrioxamine was the iron catalyst (Table 4).

DISCUSSION

The ability of paraquat to promote the oxidation of NADPH and the generation of ·OH by NADPH–cytochrome P-450 reductase was enhanced in the presence of a variety of ferric complexes. The increase in NADPH oxidation or ·OH generation was greater than the sum of the increase produced by either paraquat alone or the ferric complex alone, i.e. there was synergistic interactions between paraquat and the ferric complexes. In fact, certain ferric complexes such as ferric–ATP or –citrate or ferric ammonium sulfate were essentially inert in promoting the oxidation of NADPH or generation of ·OH in the absence of paraquat, but became active in the presence of paraquat. Ferric–ATP became nearly as active as ferric–EDTA in the presence of paraquat; ferric–EDTA is recognized as being among the most effective iron catalysts of the Haber–Weiss or Fenton reactions [18, 19, 29, 38–40].

A working scheme to accommodate aspects of these results is as follows:

$$\text{NADPH} + O_2 \xrightarrow{\text{Reductase}} \text{NADP}^+ + O_2^{\cdot-} \quad (1)$$

$$\text{NADPH} + \text{paraquat} \xrightarrow{\text{Reductase}} \text{NADP} + \text{paraquat radical} \quad (2)$$

* The reductase was kept frozen at −70° in a medium containing 20% glycerol. The final concentration of glycerol in the KMBA assay system under our reaction conditions was 68 mM. Since glycerol itself is a ·OH scavenger, we tested the competition between glycerol and KMBA for ·OH generated by a model chemical system, the iron catalyzed oxidation of ascorbate reaction system. Glycerol, at a final concentration of 68 mM, inhibited the oxidation of 1 mM KMBA by about 40%. Hence, rates of KMBA (and ethanol or other ·OH scavenger substrates) oxidation by the reductase system are underestimated because of the presence of glycerol.

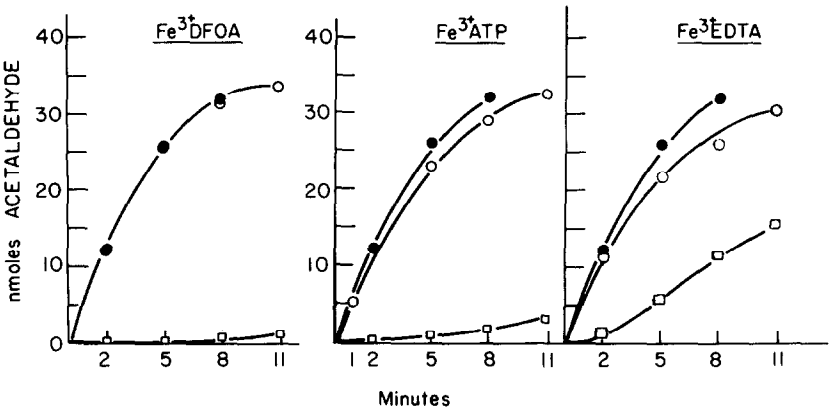

Fig. 3. Production of H_2O_2 by NADPH–cytochrome P-450 reductase. The generation of H_2O_2 was determined from the peroxidatic activity of catalase towards ethanol as described in Materials and Methods. When present, the final concentrations of ferric chelates or paraquat were 0.1 or 0.2 mM respectively. Key: (●—●) paraquat alone; (□—□) ferric chelate alone; and (○—○) paraquat plus the respective ferric chelate.

$$\text{Paraquat radical} + O_2 \rightarrow \text{paraquat} + O_2^- \quad (3)$$

$$O_2^- + O_2^- \xrightarrow{2H^+} H_2O_2 + O_2 \quad (4)$$

$$\text{Paraquat radical} + H_2O_2 \xrightarrow{(Fe?)} {}^{\cdot}OH + OH^- + \text{paraquat} \quad (5)$$

$$\text{NADPH} + Fe^{3+}\text{-complex} \xrightarrow{\text{Reductase}} \text{NADP}^+ + Fe^{2+}\text{-complex} \quad (6)$$

$$Fe^{2+}\text{-complex} + O_2 \rightarrow Fe^{3+}\text{-complex} + O_2^- \quad (7)$$

$$Fe^{2+}\text{-complex} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}\text{-complex} \quad (8)$$

$$Fe^{3+}\text{-complex} + \text{paraquat radical} \rightarrow Fe^{2+}\text{-complex} + \text{paraquat} \quad (9)$$

Reaction (1) is extremely slow as there was virtually no or little oxidation of NADPH by the reductase in the absence of any electron acceptor. The ability of paraquat alone to stimulate NADPH oxidation and production of H_2O_2 can be explained by the reaction sequences (2) to (4). There is increasing evidence that reaction (5) does not occur to a significant extent in the absence of iron [9, 11–13, 15, 17]. Under our reaction conditions, paraquat did stimulate, to a limited extent, the production of ethylene from KMBA and of acetaldehyde from ethanol in the absence of *added* iron. Paraquat itself does not bind di- or trivalent metal cations [14]. Although the phosphate buffer and the water utilized to prepare all solutions were passed through resins containing Chelex-100, small amounts of iron (estimated at about 0.5 to 2 μM [41]) are present in the reaction system as evident from the stimulation of ·OH generation by the addition of EDTA or DTPA itself [29, 42, 43]. Since ferric ammonium sulfate itself enhanced the ability of paraquat to catalyze ·OH generation by the reductase, the low production of ·OH found in the presence of paraquat (reaction 5) may involve the presence of trace amounts of iron in the reaction system. Similar considerations have been discussed by Sutton and Winterbourne [18, 19] who provided evidence against a role for reaction (5) in explaining the increase in ·OH generation by paraquat.

Reaction (6) occurs with certain ferric complexes such as ferric–EDTA or –DTPA, as shown by the stimulation of NADPH oxidation, but not to any significant extent with others such as ferric–ATP or –citrate or ferric ammonium sulfate. In view of this ability to serve as an electron acceptor from the reductase, ferric–EDTA and –DTPA complexes promoted the generation of ·OH by reductase and microsomes (Tables 3 and 4; [28, 29, 43]), whereas the other ferric complexes were relatively ineffective in this regard. The production of ·OH is likely due to reactions (6) to (8) since ·OH generation is sensitive to catalase [which blocks reaction (8) by removal of the H_2O_2 precursor] and to desferrioxamine [which is essentially inert for reaction (8)]. In view of the lack of effect of superoxide dismutase, an iron-catalyzed Haber–Weiss (superoxide-driven Fenton reaction) reaction is not the mechanism for generation of ·OH [Fe^{3+}-complex + $O_2^- \rightarrow Fe^{2+}$-complex + O_2, followed by reactions (4) and (8)].

Recent studies by Vile *et al.* [44] have shown that, under anaerobic conditions, ferric–ADP and ferric–citrate catalyze the production of ·OH from H_2O_2 plus paraquat radicals generated radiolytically or enzymatically. The increasing effectiveness of ferric–ATP or –citrate or ferric ammonium sulfate to catalyze production of ·OH in the presence of paraquat plus NADPH–cytochrome P-450 reductase under aerobic conditions is likely explained by reaction (9), in which the ferric complex is reduced by the paraquat radical, followed by reaction (8). A similar mechanism [reaction (9)] is the likely explanation for the synergism in ·OH production found with paraquat and ferric–EDTA or –DTPA. Paraquat radical rapidly reduces ferric–EDTA and ferric–DTPA [18]. In all cases, ·OH generation was very

sensitive to catalase, implicating a critical role for H_2O_2 as the precursor of ·OH (and eliminating a direct reaction between paraquat radical and the ·OH scavenger substrate), and continued to remain insensitive to superoxide dismutase [eliminating a role for enhanced O_2^- production due to reaction (3) followed by a Haber–Weiss type of reaction]. However, as a result of increased NADPH oxidation, enhanced O_2^- generation followed by increasing H_2O_2 production (reactions 3 and 4) can contribute to the synergism observed between paraquat and ferric complexes in view of the critical precursor role for H_2O_2 in reaction (8). The effectiveness of reaction (9) is evident from results showing the poor ability of desferrioxamine to prevent significantly rapid Fenton cycling between iron and H_2O_2 and paraquat radical [9], and the strong reducing potential of paraquat radical allows reduction of ferric chelates to occur at rates almost as rapid as the reaction of paraquat radical with O_2 [45]. Indeed, under our reaction conditions, enhanced production of ·OH could be observed under aerobic conditions probably because of the ability of reaction (9) to compete at least to some extent with reaction (3). In studies by Winterbourne and Sutton [19], paraquat radical generated enzymatically, but not radiolytically, was found to be capable of catalyzing ·OH production in the presence of O_2, although rates were about 10–15% those found under anaerobic conditions. These authors concluded that, although radical-driven Fenton reactions are inhibited by O_2, they do occur at physiological concentrations of O_2, especially in the presence of relatively high concentrations of added iron complexes such as those employed in the above studies.

Taken as a whole, the ability of low concentrations of ferric complexes to synergistically enhance the consumption of NADPH and the generation of ·OH from the interaction of paraquat with the reductase may contribute to the oxidative stress and toxicity produced by paraquat in cellular systems containing this or similar flavin enzymes. The synergistic interactions between reductase, paraquat and ferric complexes in generating ·OH are sensitive to catalase but not to superoxide dismutase; hence, the latter enzyme may not be protective against paraquat-mediated oxidative stress when catalyzed by this reaction system. Ferric complexes such as ferric–ATP or –citrate (or ferric–phosphate), which may be of possible physiological relevance, can become effective catalysts for the production of ·OH-like species in the presence of paraquat radical and, perhaps, other toxic agents capable of redox cycling with the reductase, e.g. quinones.

Acknowledgements—These studies were supported by U.S.P.H.S. Grant AA-03312 from the National Institute on Alcohol Abuse and Alcoholism. We thank Ms Roslyn C. King for typing the manuscript.

REFERENCES

1. Autor A, *Biochemical Mechanisms of Paraquat Toxicity*. Academic Press, New York, 1977.
2. Bus JS, Aust SD and Gibson JE, Superoxide and singlet oxygen-catalyzed lipid peroxidation as a possible mechanism for paraquat toxicity. *Biochem Biophys Res Commun* **58**: 749–755, 1974.
3. Bus JS, Aust SD and Gibson JE, Paraquat toxicity: Proposed mechanism of action involving lipid peroxidation. *Environ Health Perspect* **16**: 139–146, 1976.
4. Kappus H and Sies H, Toxic drug effects associated with oxygen metabolism; Redox cycling and lipid peroxidation. *Experientia* **37**: 1233–1241, 1981.
5. Youngman RJ and Elstner EF, Oxygen species in paraquat toxicity: The crypto-OH radical. *FEBS Lett* **129**: 265–268, 1981.
6. Bachur NR, Gordon SL, Gee MV and Kun H, NADPH–Cytochrome P-450 reductase activation of quinone anti-cancer agents to free radicals. *Proc Natl Acad Sci USA* **76**: 954–957, 1979.
7. Trush MA, Mimnaugh EG and Gram TE, Activation of pharmacologic agents to radical intermediates. *Biochem Pharmacol* **31**: 3335–3346, 1982.
8. Farrington JA, Ebert M, Land EJ and Fletcher K, Bipyridylium quaternary salts and related compounds. *Biochim Biophys Acta* **314**: 372–381, 1973.
9. Borg DC and Schaich KM, Cytotoxicity from coupled redox cycling of autoxidizing xenobiotics and metals. *Isr J Chem* **24**: 38–53, 1984.
10. Cadenas E, Brigelius R and Sies H, Paraquat induced chemiluminescence of microsomal fractions. *Biochem Pharmacol* **32**: 147–150, 1983.
11. Kohen R and Chevion M, Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. *Biochem Pharmacol* **34**: 1841–1843, 1985.
12. Kohen R and Chevion M, Transition metals potentiate paraquat toxicity. *Free Radic Res Commun* **1**: 79–88, 1985.
13. Richmond R and Halliwell B, Formation of hydroxyl radicals from the paraquat radical cation demonstrated by a highly specific gas chromatographic technique. *J Inorg Biochem* **17**: 95–107, 1982.
14. Trush MA, Mimnaugh EG, Ginsburg E and Gram TE, *In vitro* stimulation by paraquat of reactive oxygen-mediated lipid peroxidation in rat lung microsomes. *Toxicol Appl Pharmacol* **60**: 279–286, 1981.
15. Younes M, Cornelius S and Siegers CP, Ferrous-supported *in vivo* lipid peroxidation induced by compounds undergoing redox cycling. *Chem Biol Interact* **54**: 97–103, 1985.
16. Kenel MF, Bestervelt LL and Kulkarni AP, Human placental lipid peroxidation—II. NADPH and iron dependent stimulation of microsomal lipid peroxidation by paraquat. *Gen Pharmacol* **18**: 373–378, 1987.
17. Korbashi P, Kohen R, Katzhendler J and Chevion M, Iron mediates paraquat toxicity in *Escherichia coli*. *J Biol Chem* **261**: 12472–12476, 1986.
18. Sutton HC and Winterbourne CC, Chelated iron-catalyzed ·OH formation from paraquat radicals and H_2O_2; Mechanism of formate oxidation. *Arch Biochem Biophys* **235**: 106–115, 1984.
19. Winterbourne CC and Sutton HC, Hydroxyl radical production from hydrogen peroxide and enzymatically generated paraquat radicals: Catalytic requirements and oxygen dependence. *Arch Biochem Biophys* **235**: 116–126, 1984.
20. Muliawan H, Schenlen ME and Kappus H, Adriamycin stimulates only the iron-induced, NADPH-dependent microsomal alkane formation. *Biochem Pharmacol* **31**: 3147–3150, 1982.
21. Sterrenberg L, Julicher RHM, Bast A and Noordhoek J, Adriamycin stimulates NADPH-dependent lipid peroxidation in liver microsomes not only by enhancing the production of O_2^- and H_2O_2 but also by potentiating the catalytic activity of ferrous ions. *Toxicol Lett* **22**: 153–159, 1984.
22. Sugioka K and Nakano M, Mechanism of phospholipid peroxidation induced by ferric iron–ADP co-ordination complex. *Biochim Biophys Acta* **713**: 333–343, 1982.

23. Gutteridge JMC, Quinlan GJ and Wilkins S, Mitomycin C-induced deoxyribose degradation inhibited by superoxide dismutase. A reaction involving iron, hydroxyl and semiquinone radicals. *FEBS Lett* **167**: 37–41, 1984.
24. Younes M, Cornelius S and Siegers CP, Ferrous ion supported *in vivo* lipid peroxidation induced by paracetamol—Its relation to hepatotoxicity. *Res Commun Chem Pathol Pharmacol* **51**: 89–99, 1986.
25. Beloqui O and Cederbaum AI, Microsomal interactions between iron, paraquat and menadione: Effect on hydroxyl radical production and alcohol oxidation. *Arch Biochem Biophys* **242**: 187–196, 1985.
26. Koop DR, Nordblom DC and Coon MJ, Immunochemical evidence for a role of cytochrome P-450 in liver microsomal ethanol oxidation. *Arch Biochem Biophys* **235**: 228–238, 1984.
27. Morgan ET, Koop DR and Coon MJ, Catalytic activity of cytochrome P-450 isozyme 3a isolated from liver microsomes of ethanol-treated rabbits. *J Biol Chem* **257**: 13951–13957, 1982.
28. Winston GW and Cederbaum AI, NADPH-dependent production of oxy-radicals by purified components of the rat liver mixed-function oxidase system. II. Role in microsomal oxidation of ethanol. *J Biol Chem* **258**: 1514–1519, 1983.
29. Winston GW, Feierman DE and Cederbaum AI, The role of iron chelates in hydroxyl radical production by rat liver microsomes, NADPH cytochrome P-450 reductase and xanthine oxidase. *Arch Biochem Biophys* **232**: 378–390, 1984.
30. Morehouse LA, Thomas CE and Aust SD, Superoxide generation by NADPH cytochrome P-450 reductase: The effect of iron chelators and the role of superoxide in microsomal lipid peroxidation. *Arch Biochem Biophys* **232**: 365–377, 1984.
31. Cederbaum AI and Dicker E, Inhibition of microsomal oxidation of alcohols and of hydroxyl radical scavenging agents by the iron chelating agent desferrioxamine. *Biochem J* **210**: 107–113, 1983.
32. Guengerich PF and Martin MV, Purification of cytochrome P-450, NADPH cytochrome P-450 reductase and epoxide hydratase from a single preparation of rat liver microsomes. *Arch Biochem Biophys* **205**: 365–379, 1980.
33. Phillips AH and Langdon RG, Hepatic triphosphopyridine nucleotide-cytochrome *c* reductase: Isolation, characterization and kinetic studies. *J Biol Chem* **237**: 2652–2660, 1962.
34. Cederbaum AI and Cohen G, Ethanol oxidation and microsomal radical production. *Methods Enzymol* **105**: 516–522, 1984.
35. Cederbaum AI and Cohen G, Microsomal oxidation of hydroxyl radical scavenging agents. In: *Handbook of Methods for Oxygen Radical Research* (Ed. Greenwald RA), pp. 81–87. CRC Press, Boca Raton, FL, 1985.
36. Hildebrandt AG, Roots I, Tjoe M and Heinemeyer G, Hydrogen peroxide in hepatic microsomes. *Methods Enzymol* **52**: 342–350, 1978.
37. Anbar M and Neta P, A compilation of specific biomolecular constants for the reaction of hydrated electrons, hydrogen atoms and hydroxyl radicals with inorganic and organic compounds in aqueous solutions. *Int J Appl Radiat Isotopes* **18**: 493–523, 1967.
38. Halliwell B, Superoxide-dependent formation of hydroxyl radicals in the presence of iron salts. *FEBS Lett* **96**: 238–242, 1978.
39. Halliwell B and Gutteridge JMC, Oxygen toxicity, oxygen radicals, transition metals and disease. *Biochem J* **219**: 1–17, 1984.
40. McCord J and Day ED, Superoxide-dependent production of hydroxyl radical catalyzed by iron–EDTA complex. *FEBS Lett* **86**: 139–142, 1978.
41. Krikun G and Cederbaum AI, Effect of chronic ethanol consumption on microsomal lipid peroxidation; Role of iron and comparison between controls. *FEBS Lett* **208**: 292–296, 1986.
42. Feierman DE and Cederbaum AI, The effect of EDTA and iron on the oxidation of hydroxyl radical scavenging agents and ethanol by rat liver microsomes. *Biochem Biophys Res Commun* **116**: 765–770, 1983.
43. Winston GW and Cederbaum AI, NADPH-dependent production of oxy radicals by purified components of the rat liver mixed function oxidase system. I. Oxidation of hydroxyl radical scavenging agents. *J Biol Chem* **258**: 1508–1513, 1983.
44. Vile GF, Winterbourne CC and Sutton HC, Radical-driven Fenton reactions: Studies with paraquat, adriamycin, and anthraquinone 6-sulfonate and citrate, ATP, ADP, and pyrophosphate iron chelates. *Arch Biochem Biophys* **259**: 616–626, 1987.
45. Land EJ and Swallow AJ, Electron transfer from pyridinyl radicals to cytochrome *c*. *Ber Bunsenges Phys Chem* **79**: 436–437, 1975.