Biochemical Pharmacology, Vol. 38, No. 17, pp. 2911–2918, 1989.
Printed in Great Britain.

0006-2952/89 $3.00 + 0.00

INTERACTIONS BETWEEN PARAQUAT AND FERRIC COMPLEXES IN THE MICROSOMAL GENERATION OF OXYGEN RADICALS

SUSANA PUNTARULO and ARTHUR I. CEDERBAUM*
Department of Biochemistry, Mount Sinai School of Medicine, New York, NY 10029, U.S.A.

(*Received* 17 *October* 1988; *accepted* 18 *January* 1989)

Abstract—Transition metals may play a central role in the toxicity associated with paraquat. Studies were carried out to evaluate the interaction of paraquat with several ferric complexes in the promotion of oxygen radical generation by rat liver microsomes. In the absence of *added* iron, paraquat produced some increase in low level chemiluminescence by microsomes; there was a synergistic increase in light emission in the presence of paraquat plus ferric–ATP or ferric–citrate, but not paraquat plus either ferric–EDTA or ferric–diethylenetriamine pentaacetic acid (ferric–DETAPAC). Synergistic interactions could be observed at a paraquat concentration of 100 μM and a ferric–ATP concentration of 3 μM. In the absence or presence of paraquat, microsomal light emission was not affected by catalase or dimethyl sulfoxide (DMSO), indicating no significant role for hydroxyl radicals. Superoxide dismutase (SOD) did not affect chemiluminescence in the absence of paraquat but produced some inhibition in the presence of paraquat; this inhibition by SOD was most prominent in the absence of added iron and less pronounced in the presence of ferric–ATP or ferric–citrate. Although microsomal chemiluminescence is closely associated with lipid peroxidation, paraquat did not increase malondialdehyde production as reflected by production of thiobarbituric acid-reactive components. However, lipid peroxidation was sensitive to inhibition by SOD in the presence, but not in the absence, of paraquat, analogous to results with chemiluminescence. Paraquat synergistically increased microsomal hydroxyl radical production as measured by the production of ethylene from 2-keto-4-thiomethylbutyrate in the presence of ferric–EDTA or ferric–citrate. The interaction of paraquat with microsomes and ferric complexes resulted in an increase in oxygen radical generation. Various ferric complexes can increase the catalytic effectiveness of paraquat in promoting microsomal generation of oxygen radicals, although, depending on the reaction being investigated, the nature of the ferric complex is important.

The toxicity associated with paraquat has generally been attributed to the generation of reactive oxygen intermediates [1–5]. Bus *et al.* [2, 3] originally proposed that paraquat increased microsomal lipid peroxidation. A variety of studies have supported the concept that paraquat causes an increase in lipid peroxidation [2, 6–10]; however, others have not confirmed this [11–16]. Incubation of microsomes or purified NADPH–cytochrome P-450 reductase with paraquat results in an increase in NADPH and oxygen utilization, and the subsequent production of superoxide anion radical and H_2O_2 [2, 3, 6]. It appears that for the generation of more potent oxidants such as species with the oxidizing power of the hydroxyl radical (·OH) or which can initiate lipid peroxidation, transition metals such as iron or copper are required. While several studies have reported promotion of microsomal lipid peroxidation (malondialdehyde generation) in the absence of added iron, other studies have demonstrated that added iron salts are necessary to promote the toxicity associated with paraquat and other redox cycling agents [17–23].

Since the effects of paraquat on rat liver microsomal lipid peroxidation are complex, studies were conducted to compare the ability of paraquat to promote the generation of low level chemiluminescence by microsomes to its effect on microsomal lipid peroxidation and ·OH generation. In view of the important role for iron in catalyzing these reactions, the interaction of paraquat with several different iron complexes to generate reactive oxygen intermediates was evaluated. In microsomal systems, certain ferric complexes such as ferric–EDTA or ferric–diethylenetriamine pentaacetic acid (ferric–DETAPAC) are very effective catalysts for ·OH generation but are inhibitory towards lipid peroxidation, whereas other ferric complexes such as ferric–ATP and ferric–citrate show the opposite catalytic effectiveness [24–27]. It therefore appeared important to attempt to define the interaction of paraquat with different iron chelates in catalyzing microsomal generation of potent oxidizing radicals such as ·OH and the species responsible for initiating chemiluminescence and lipid peroxidation.

MATERIALS AND METHODS

Liver microsomes were isolated from male, Sprague–Dawley rats weighing about 170–200 g. The livers were perfused with 0.9% NaCl and homogenized in a solution containing 0.25 M sucrose–0.01 M Tris–HCl (pH 7.4)–0.001 M EDTA–50 μM desferrioxamine. The microsomes were isolated by differential centrifugation, washed twice with

* Correspondence: Dr Arthur I. Cederbaum, Department of Biochemistry, Box 1020, Mount Sinai School of Medicine, One Gustave L. Levy Place, New York, NY 10029, U.S.A.

0.125 M KCl, and stored at −70°.

The basic reaction system to assay for chemiluminescence consisted of 100 mM potassium phosphate, pH 7.4, 10 mM $MgCl_2$, 0.4 mM $NADP^+$, 10 mM glucose-6-phosphate and 1.4 units of glucose-6-phosphate dehydrogenase in a final volume of 3 ml. Reactions were carried out essentially as previously described [28] in 13 × 40 mm tubes which were then placed inside glass scintillation vials, which were previously dark-adapted. The reactions were initiated by the addition of approximately 0.5 mg of microsomal protein, and chemiluminescence was measured at room temperature over a 5-min time period in a Beckman LS-230 liquid scintillation counter in the out-of-coincidence mode with the discriminator adjusted for low level emission. The background level of the empty tubes and vials was subtracted from all experimental results. Since the chemiluminescence response is non-linear, quantitation was performed by cutting out the appropriate curves and weighing their areas; results are expressed as arbitrary units.

Microsomal lipid peroxidation was determined by assaying for the rate of production of thiobarbituric acid-reaction components (expressed as malondialdehyde equivalents) [29]. The absorbance of the supernatant fraction was determined at a wavelength of 535 nm, and the concentration of malondialdehyde was calculated using an extinction coefficient of 156 mM/cm [30].

Microsomal generation of hydroxyl radical or species with the oxidizing power of the hydroxyl radical was determined by assaying for the generation of ethylene gas from 2-keto-4-thiomethylbutyric acid (KMB). The basic system was similar to that utilized to measure chemiluminescence except that the final volume was 1 ml, microsomal protein was about 1 mg, and 1 mM azide was present to inhibit catalase activity present in the isolated microsomal fractions. Experiments were conducted essentially as previously described [31]. All reaction systems were characterized for their dependence on microsomes, NADPH and O_2. In general, omission of any of these resulted in a 80–95% decrease in chemiluminescence, lipid peroxidation or ·OH generation.

The ferric complexes were prepared by dissolving ferric ammonium sulfate in 0.1 N HCl, and then diluting with the respective chelator to the appropriate stock concentration. Ferric–ATP was utilized as a 1:20 complex, whereas the other ferric complexes were all utilized as a 1:2 complex. The oxidation of NADPH by microsomes was determined from the decrease in absorbance at 340 nm. All reagents were of the highest grade available. The buffers, and the water used to prepare all solutions were passed through columns containing Chelex-100 resin to remove metal contaminants. All results are from experiments carried out in duplicate, and replicated with at least two different microsomal preparations. Where indicated, values refer to mean ± SE.

RESULTS

Effect of paraquat on microsomal chemiluminescence. The effect of paraquat on microsomal light emission was determined in the absence and presence of added ferric–citrate. In the absence of added iron, paraquat produced some increase in chemiluminescence (Fig. 1A) which was variable from preparation to preparation, most likely as a consequence of varying amounts of iron present in the microsomal preparation. A more consistent increase by paraquat was obtained in the presence of ferric–citrate (Fig. 1B); only short reaction time periods were utilized in these experiments since the reaction system rapidly became anaerobic in the presence of paraquat. The elevated light emission produced during the first minute of reaction was probably due to preformed lipid hydroperoxides in the microsomal preparation. The ability of paraquat to stimulate microsomal chemiluminescence in the absence or presence of added ferric–citrate was linear with microsomal protein, up to about 0.2 mg/ml (Fig. 1C).

Previous experiments indicated that ferric complexes such as ferric–ATP or ferric–citrate increase microsomal chemiluminescence, whereas complexes such as ferric–EDTA or ferric–DETAPAC are inhibitory [28]. The different effects of the iron complexes most likely reflect the close association between microsomal chemiluminescence and lipid peroxidation [32–36] (processes increased by ferric–ATP or ferric–citrate) and the lack of association with microsomal ·OH generation (increased by ferric–EDTA or ferric–DETAPAC) [24, 25]. Paraquat increased microsomal light emission in the absence and presence of added ferric complexes; however, the extent of increase was more notable in the presence of ferric–ATP or ferric–citrate (Table 1). The last column of Table 1 shows the net increase in microsomal chemiluminescence produced by paraquat. In the presence of ferric–ATP or ferric–citrate, paraquat was more effective in stimulating microsomal light emission than in the absence of added iron, whereas it was somewhat less effective in the presence of ferric–EDTA or ferric–DETAPAC.

A concentration curve for the ability of paraquat to stimulate microsomal chemiluminescence in the absence and presence of ferric–ATP is shown in Fig. 2. Light emission increased as the concentration of paraquat was elevated over the range of 25 to 500 μM. A more pronounced concentration dependence was observed in the presence of ferric–ATP. The inset of Fig. 2 shows the synergistic increase in chemiluminescence produced by the combinations of paraquat plus ferric–ATP over that produced by paraquat itself; a maximal stimulatory effect was obtained at a paraquat concentration of 100 μM when the concentration of added iron was held constant at 50 μM.

A concentration curve for the ability of ferric–ATP to stimulate microsomal chemiluminescence in the absence and presence of paraquat is shown in Table 2. Light emission increased as the concentration of ferric–ATP increased. A synergistic increase in chemiluminescence by 100 μM paraquat could be observed at an iron concentration of 3 μM and was maximal at 25 μM ferric–ATP (Table 2). Essentially similar results were obtained with ferric–citrate as the iron catalyst (data not shown).

Effect of radical scavengers on microsomal chemi-

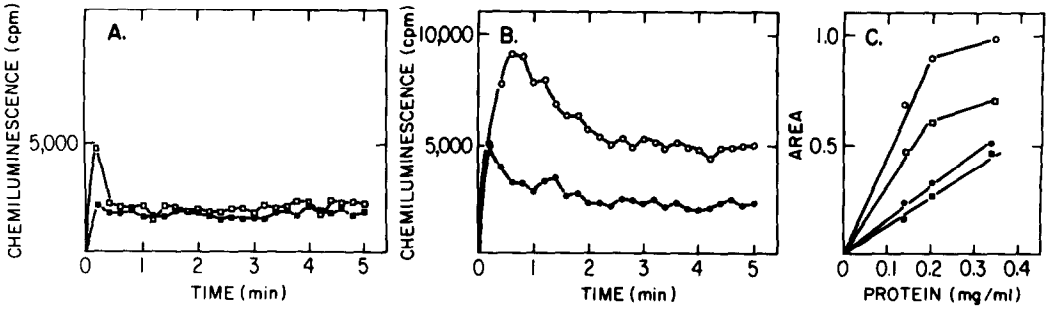

Fig. 1. Effect of paraquat on NADPH-dependent chemiluminescence by microsomes. Reactions were carried out as described under Materials and Methods. Panel A: (■) no added iron; (□) no added iron plus 0.1 mM paraquat. Panel B: (●) 50 μM ferric–citrate; (○) 50 μM ferric–citrate plus 0.1 mM paraquat. Microsomal protein was 0.17 mg/ml. Panel C: microsomal protein was varied as indicated, and light emission was determined over a 5-min reaction period. Key: (■) no added iron; (□) no added iron plus 0.1 mM paraquat; (●) 50 μM ferric–citrate; (○) 50 μM ferric–citrate plus 0.1 mM paraquat. Results are expressed as area in arbitrary units.

Table 1. Effect of iron and paraquat on microsomal chemiluminescence

Added ferric complex	Chemiluminescence (area/5 min)		
	−Paraquat	+Paraquat	Net increase by paraquat
None	0.136 ± 0.025	0.237 ± 0.030	0.091 ± 0.033
Fe–citrate	0.221 ± 0.031	0.504 ± 0.040	0.285 ± 0.071
Fe–ATP	0.214 ± 0.033	0.431 ± 0.025	0.202 ± 0.003
Fe–EDTA	0.114 ± 0.012	0.179 ± 0.033	0.066 ± 0.020
Fe–DETAPAC	0.111 ± 0.007	0.189 ± 0.026	0.078 ± 0.026

Microsomal chemiluminescence was assayed over a 5-min reaction period in the presence of the indicated ferric complexes (50 μM final concentration of iron) and in the absence or presence of 0.1 mM paraquat. Results (mean ± SE) are from four microsomal preparations.

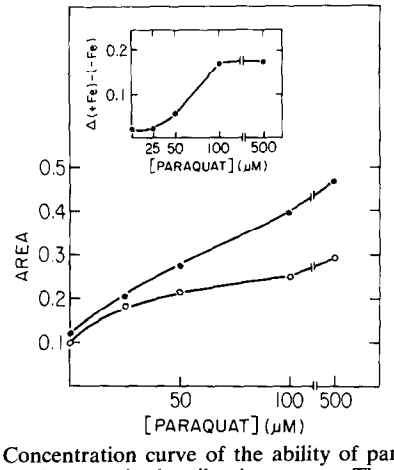

Fig. 2. Concentration curve of the ability of paraquat to increase microsomal chemiluminescence. The concentration of paraquat was varied as indicated, and light emission was determined over a 5-min reaction period in the absence of added iron (○) or in the presence of 50 μM ferric–ATP (●). The inset shows the difference between the areas in the absence and presence of ferric–ATP as a function of the concentration of paraquat.

luminescence. Light emission by microsomes in the absence or presence of added iron was not affected significantly by either superoxide dismutase, catalase or the ·OH scavenger dimethyl sulfoxide (DMSO) (Table 3). In the presence of paraquat, chemiluminescence was again not affected by catalase or by DMSO; however, some inhibition by superoxide dismutase was noted. The net increase in light emission produced by the addition of paraquat is shown in the last column of Table 3. In the absence or presence of added iron, a decrease in the paraquat-stimulated chemiluminescence was produced by superoxide dismutase but not by catalase or DMSO.

Effect of paraquat and iron on microsomal NADPH oxidation. Ferric–EDTA and –DETAPAC increased microsomal NADPH oxidation, whereas ferric–ATP and ferric–citrate were without effect (Table 4). This is in accord with previous results showing that the former two ferric complexes, but not the latter two, were effective electron acceptors from NADPH–cytochrome P-450 reductase [24, 26]. Paraquat, as had been observed by others [2, 3, 6, 8], increased NADPH oxidation by the microsomes; the net increase in NADPH consumption produced

Table 2. Concentration curve for the stimulation of microsomal chemiluminescence by ferric–ATP

Concentration of ferric–ATP (μM)	Chemiluminescence (area/5 min)		
	−Paraquat	+Paraquat	Net increase by paraquat
0	0.132	0.212	0.088
3	0.172	0.341	0.169
10	0.207	0.432	0.225
25	0.233	0.579	0.346
50	0.263	0.588	0.325

Microsomal chemiluminescence was determined in the presence of the indicated concentrations of ferric–ATP (1:20 chelate) and in the absence or presence of 0.1 mM paraquat. Results are from two microsomal preparations.

Table 3. Effect of radical scavenging agents on microsomal chemiluminescence

Added ferric complex	Addition	Chemiluminescence (area/5 min)		
		−Paraquat	+Paraquat	Net increase by paraquat
None	—	0.173 ± 0.053	0.365 ± 0.061	0.192 ± 0.006
	SOD	0.187 ± 0.031 (+8)	0.246 ± 0.055 (−33)	0.059 ± 0.012 (−70)
	Catalase	0.155 ± 0.006 (−10)	0.375 ± 0.027 (+3)	0.220 ± 0.017 (+14)
	DMSO	0.242 ± 0.007 (+39)	0.438 ± 0.061 (+20)	0.196 ± 0.034 (+2)
Fe–citrate	—	0.223 ± 0.011	0.556 ± 0.019	0.333 ± 0.031
	SOD	0.214 ± 0.010 (−4)	0.446 ± 0.012 (−20)	0.232 ± 0.009 (−30)
	Catalase	0.135 ± 0.021 (−39)	0.499 ± 0.064 (−10)	0.364 ± 0.085 (+9)
	DMSO	0.268 ± 0.039 (+20)	0.608 ± 0.043 (+9)	0.399 ± 0.082 (+19)

Microsomal chemiluminescence was determined in the presence of the indicated ferric complexes (50 μM final concentration) and in the absence or presence of 0.1 mM paraquat. When present, the final concentrations of SOD and catalase were 3 units/ml and 65 units/ml, respectively, and that of DMSO was 50 mM. Results (mean ± SE) are from three microsomal preparations. Numbers in parentheses refer to the effect of the addition on the appropriate control rate. Essentially, similar results were obtained with ferric–ATP in place of ferric–citrate as the iron catalyst, e.g. the net increase in chemiluminescence produced by paraquat in the presence of ferric–ATP was affected as follows: SOD, −33%; catalase, +17%; DMSO, −7%.

Table 4. Effect of iron and paraquat on microsomal oxidation of NADPH

Added ferric complex	Rate of NADPH oxidation (μmol/min/mg protein)		
	−Paraquat	+Paraquat	Net increase by paraquat
None	0.030 ± 0.010	0.048 ± 0.018	0.018 ± 0.007
Fe–citrate	0.034 ± 0.012	0.056 ± 0.019	0.020 ± 0.008
Fe–ATP	0.019 ± 0.007	0.035 ± 0.011	0.017 ± 0.004
Fe–EDTA	0.070 ± 0.015	0.074 ± 0.015	0.004 ± 0.001
Fe–DETAPAC	0.236 ± 0.041	0.238 ± 0.041	0.002 ± 0.001

The oxidation of 0.2 mM NADPH by microsomes was determined in the presence of the indicated ferric complexes (50 μM final concentration) and in the absence or presence of 0.1 mM paraquat. Results (mean ± SE) are from three microsomal preparations.

by paraquat was the same in the absence of added iron or in the presence of ferric–citrate and ferric–ATP (Table 4). However, paraquat did not produce an increase in NADPH oxidation over the already augmented rates found in the presence of ferric–EDTA or ferric–DETAPAC (Table 4).

Effect of paraquat on microsomal lipid peroxidation. The effect of paraquat in the absence and presence of added iron on microsomal lipid peroxidation (production of TBA-reactive material) was determined. Microsomal lipid peroxidation was increased in the presence of ferric–ATP or ferric–

Table 5. Effect of iron and paraquat on microsomal lipid peroxidation

Added ferric complex	Lipid peroxidation (nmol TBA-reactive material/min/mg protein)		
	−Paraquat	+Paraquat	Effect of paraquat (%)
None	4.22 ± 0.05	3.78 ± 0.70	−10
Fe–citrate	7.79 ± 0.19	8.64 ± 0.44	+11
Fe–ATP	6.70 ± 0.54	7.27 ± 0.81	+9
Fe–EDTA	1.36 ± 0.24	1.21 ± 0.36	−11

Microsomal lipid peroxidation was determined as described under Materials and Methods in the presence of the indicated ferric complexes (50 μM final concentration) and in the absence or presence of 0.1 mM paraquat. Results (mean ± SE) are from four microsomal preparations.

citrate but decreased by ferric–EDTA (Table 5) and nearly abolished in the presence of ferric–DETAPAC (data not shown). Paraquat had no significant effect on the total rate of microsomal lipid peroxidation; actually, a trend towards a slight decrease in lipid peroxidation was produced by paraquat in the absence of added iron whereas no such decrease or a slight net increase was produced by paraquat in the presence of ferric–ATP or ferric–citrate (Table 5; results also in Table 6). The changes, although very small, were consistently observed from preparation to preparation.

Microsomal lipid peroxidation is not sensitive to either superoxide dismutase, catalase or ·OH scavengers [24, 25]. Results in Table 6 confirm this for experiments carried out in the absence of added iron or the presence of ferric–citrate. In the presence of paraquat, lipid peroxidation remained insensitive to catalase and DMSO. However, some inhibition by superoxide dismutase could now be observed (Table 6), analogous to the inhibition by superoxide dismutase of chemiluminescence in the presence of paraquat (Table 3). Column 5 of Table 6 shows the net change in lipid peroxidation produced by paraquat. In the absence of added iron, paraquat decreased lipid peroxidation, and there was a further decrease in the presence of SOD (but not in the presence of catalase or DMSO). The percent decreases of lipid peroxidation produced by paraquat (last column of Table 6) were similar in the absence or presence of catalase and DMSO, but were decreased further in the presence of SOD. In the presence of ferric–citrate, lipid peroxidation was not decreased by paraquat, but was decreased in the presence of paraquat plus SOD over the corresponding rate in the presence of SOD alone (Table 6; columns 5 and 6). Lipid peroxidation in the presence of paraquat remained insensitive to catalase or DMSO, similar to results in the absence of paraquat.

Effect of paraquat on hydroxyl radical production by microsomes. For comparative purposes, the effect of paraquat on microsomal generation of ·OH was evaluated by determining the production of ethylene from 2-keto-4-thiomethylbutyric acid. In the absence of added iron, the microsomes catalyzed a low rate of production of ethylene (Fig. 3). Ferric–EDTA increased ethylene production, whereas ferric–citrate was not especially effective as a catalyst for ·OH production. In the absence or presence of added iron, ethylene production was elevated 2- to 3-fold by the addition of paraquat (Fig. 3). This increase in iron-catalyzed ·OH generation produced by paraquat was sensitive to inhibition by catalase but not by superoxide dismutase (data not shown).

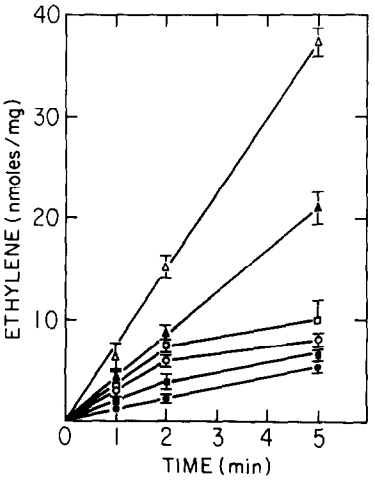

Fig. 3. Effect of paraquat on microsomal ·OH generation. The oxidation of 10 mM KMB to ethylene gas was determined under the following conditions: (●) no added iron; (○) no added iron plus 0.1 mM paraquat; (■) 50 μM ferric–citrate; (□) 50 μM ferric–citrate plus 0.1 mM paraquat; (▲) 50 μM ferric–EDTA; (△) 50 μM ferric–EDTA plus 0.1 mM paraquat.

DISCUSSION

The interaction of paraquat with microsomes, in the presence of certain ferric complexes, results in an increase in oxygen radical generation as reflected by increases in low level chemiluminescence and ·OH generation. Ferric complexes increase the catalytic effectiveness of paraquat in generating reactive oxygen intermediates, as the net increases in chemiluminescence or ·OH production produced in the presence of paraquat plus iron was greater than the sum of the increase produced by paraquat alone plus ferric complex alone. Previous results have shown that ferric–EDTA and –DETAPAC complexes are excellent catalysts for microsomal ·OH

Table 6. Effect of radical scavenging agents on microsomal lipid peroxidation

Added ferric complex	Scavenger	Lipid peroxidation (nmol/min/mg protein) −Paraquat	+Paraquat	Net change by paraquat	Effect by paraquat (%)
None	—	2.28 ± 0.36	1.46 ± 0.42	−0.82	−36
	SOD	1.95 ± 0.31 (−14)	0.72 ± 0.25 (−51)	−1.23	−63
	Catalase	2.04 ± 0.20 (−10)	1.14 ± 0.45 (−21)	−0.90	−44
	DMSO	2.15 ± 0.17 (−6)	1.26 ± 0.35 (−14)	−0.89	−41
Fe–citrate	—	3.34 ± 0.09	3.42 ± 0.07	+0.08	+2
	SOD	2.55 ± 0.60 (−23)	2.03 ± 0.15 (−41)	−0.52	−20
	Catalase	2.59 ± 0.35 (−22)	3.37 ± 0.03 (−2)	+0.78	+30
	DMSO	2.99 ± 0.31 (−11)	3.55 ± 0.08 (+4)	+0.56	+19

Microsomal lipid peroxidation was determined in the absence or presence of 50 μM ferric–citrate and/or 0.1 mM paraquat. When present, final concentrations of SOD and catalase were 3 units/ml and 65 units/ml, respectively, and that of DMSO was 50 mM. Results (means ± SE) are from three microsomal preparations. Numbers in parentheses refer to the effect of SOD or catalase or DMSO on the respective control rate of lipid peroxidation.

generation, whereas ferric–ATP or –citrate are poor catalysts [26, 27]; microsomal chemiluminescence is increased with the latter ferric complexes but is decreased with ferric–EDTA or –DETAPAC [28]. Dissociation constants for the various ferric complexes utilized are: ferric–EDTA, 10^{22}–10^{25}; ferric–DETAPAC, $10^{28.6}$; ferric–citrate, 10^{12}–10^{16} [37–40]; while that for ferric–ATP is dependent on pH, the ratio of iron to ATP, and formation of polynuclear complexes, but has been reported to be about 10^{6} [41] or even to be as high as that for ferric–citrate [42]. Rates of production of TBA-reactive material and light emission appear to correlate inversely with the dissociation constants, whereas ·OH generation appears to correlate directly. A similar trend in catalytic effectiveness of the ferric complex was observed in the presence of paraquat; for example, chemiluminescence was synergistically increased in the presence of paraquat plus either ferric–ATP or ferric–citrate, as compared to the rates in the absence of added iron, whereas no increase was found in the presence of paraquat plus either ferric–EDTA or ferric–DETAPAC. Hydroxyl radical generation was increased to a similar small extent by paraquat in the absence of added iron or in the presence of ferric–EDTA. Thus, although paraquat synergistically interacts with the appropriate ferric complexes to increase their catalytic effectiveness in generating reactive oxygen intermediates, paraquat does not change the rank order of effectiveness of the ferric complexes in stimulating production of that particular oxygen radical species.

Paraquat radical readily reduces iron complexes such as ferric–EDTA or –DETAPAC [21]; the reducing power of paraquat radical is such that ferric chelates are reduced almost as rapidly as paraquat radical reacts with oxygen [43]. The increase in microsomal ·OH generation produced by paraquat may therefore reflect an increased rate of reduction of the ferric complex to ferrous, followed by a Fenton-type of reaction to yield ·OH. Alternatively, reaction of paraquat radical with O_2 to yield superoxide radical, followed by dismutation can result in increased production of H_2O_2, the precursor of ·OH. The latter possibility may be more relevant since the high rates of NADPH consumption in the presence of ferric–EDTA or –DETAPAC were not increased further by paraquat (Table 4).

In view of the lack of sensitivity to catalase which indicates that H_2O_2 is not an intermediate in the production of the oxidant responsible for chemiluminescence, the increase in light emission produced by paraquat may be due to an increased rate of reduction of the ferric–ATP or ferric–citrate catalyst. Cadenas *et al.* [44] originally reported that SOD (but not catalase) inhibited microsomal light emission in the presence of paraquat and proposed a mechanism in which the dismutation of $O_2^{\cdot-}$, derived from the interaction of paraquat radical with O_2, would yield chemiluminescence via production of singlet oxygen. Our results indicated that, whereas SOD had little effect on chemiluminescence in the absence of paraquat, in agreement with the data of Cadenas *et al.* [44], SOD (but not catalase or DMSO) produced inhibition of light emission in the presence of paraquat (Table 3). This inhibition by SOD was most notable in the absence of added iron. Low level chemiluminescence generally results from the decay of excited carbonyl species and from singlet oxygen decay [45–47]. In the absence of added iron, singlet oxygen production, perhaps from the dismutation of $O_2^{\cdot-}$ (arising via paraquat radical interaction with oxygen), may be primarily responsible for the light emission [44] whereas in the presence of added iron, enhanced reduction of the ferric complex by paraquat radical and subsequent formation of the initiating ferrous-oxidant plus decomposition of preformed hydroperoxides may lead to light emission primarily via decay of excited carbonyl species. The former reaction sequence would be more sensitive to SOD than the latter.

The effect of paraquat on microsomal lipid peroxidation is complex, with varying reports of an increase in lipid peroxidation, no change, or even a decrease [2, 3, 6–18]. The processes of microsomal lipid peroxidation and chemiluminescence are closely associated with each other [32–36]. Under conditions in which paraquat increased microsomal

chemiluminescence, it did not produce any significant increase in the amount of thiobarbituric acid reactive material, which was taken as a measure of lipid peroxidation. In view of the increase in chemiluminescence, the lack of an increase in TBA-reactive material may reflect interaction of paraquat or paraquat radical with an intermediate required for the ultimate production of malondialdehyde but not for light emission, i.e. paraquat may increase a step required for initiation of lipid peroxidation and chemiluminescence, followed by a quenching of a propagation step required for producing malondialdehyde. The varying reported effects of paraquat on lipid peroxidation may be due, in part, to the absence or presence of added iron, the chelate utilized, and the concentration of the iron, which may help to dictate whether an initiating, propagating or termination step is limiting for malondialdehyde production. In support of these considerations are the results indicating that paraquat decreases lipid peroxidation in the absence of added iron but not in the presence of added iron (Tables 5 and 6).

Although the situation with regard to lipid peroxidation remains complex, the ability of paraquat to increase microsomal light emission and $^{\cdot}OH$ generation indicates that paraquat does increase the production of potent oxidants by microsomes, and that this increase requires the presence of appropriately chelated iron. Various ferric complexes can increase the catalytic effectiveness of paraquat in promoting oxygen radical generation by microsomes in a synergistic manner, although depending on the reaction under investigation the nature of the ferric complex is important. The ability of ferric complexes such as ferric–ATP or –citrate to synergistically elevate the production of reactive oxygen intermediates from the interaction of paraquat with microsomes may contribute to the oxidative stress and toxicity produced by paraquat in biological systems.

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. 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.
7. Aldrich TK, Fisher AB, Cadenas E and Chance B, Evidence for lipid peroxidation by paraquat in the perfused rat lung. *J Lab Clin Med* **101**: 66–73, 1983.
8. Trush MA, Mimnaugh EG, Ginsburg E and Gram TE, Studies on the *in vitro* interaction of mitomycin C, nitrofurantoin and paraquat with pulmonary microsomes. Stimulation of reactive oxygen-dependent lipid peroxidation. *Biochem Pharmacol* **31**: 805–814, 1982.
9. Reddy KA, Litov RE and Omaye ST, Effect of pretreatment with antiinflammatory agents on paraquat toxicity in rat. *Res Commun Chem Pathol Pharmacol* **17**: 87–100, 1977.
10. Burk RF, Lawrence RA and Lane JM, Liver necrosis and lipid peroxidation in the rat as a result of paraquat and diquat administration. *J Clin Invest* **65**: 1024–1031, 1980.
11. Ilett KF, Stripp B, Menard RH, Reid WD and Gillette JR, Studies on the mechanism of the lung toxicity of paraquat. *Toxicol Appl Pharmacol* **28**: 216–226, 1974.
12. Steffen C and Netter KJ, On the mechanism of paraquat action in microsomal oxygen reduction and its relation to lipid peroxidation. *Toxicol Appl Pharmacol* **47**: 593–602, 1979.
13. Kornbrust DJ and Mavis RD, The effect of paraquat on microsomal lipid peroxidation *in vitro* and *in vivo*. *Toxicol Appl Pharmacol* **53**: 323–332, 1980.
14. Misra HP and Gorsky LD, Paraquat and NADPH-dependent lipid peroxidation in lung microsomes. *J Biol Chem* **256**: 9994–9998, 1981.
15. Steffen C, Muliawan H and Kappus H, Lack of *in vivo* lipid peroxidation in experimental paraquat poisoning. *Naunyn-Schmiedebergs Arch Pharmacol* **310**: 241–243, 1980.
16. Sandy MS, Moldeus P, Ross D and Smith MT, Role of redox cycling and lipid peroxidation in bipyridyl herbicide toxicity. Studies with a compromised isolated hepatocyte model system. *Biochem Pharmacol* **35**: 3095–3101, 1986.
17. Borg DC and Schaich KM, Cytotoxicity from coupled redox cycling of autoxidizing xenobiotics and metals. *Isr J Chem* **24**: 38–53, 1984.
18. Kohen R and Chevion M, Paraquat toxicity is enhanced by iron and reduced by desferrioxamine in laboratory mice. *Biochem Pharmacol* **34**: 1841–1843, 1985.
19. Kohen R and Chevion M, Transition metals potentiate paraquat toxicity. *Free Radic Res Commun* **1**: 79–88, 1985.
20. Younes M, Cornelius S and Siegers CP, Ferrous iron supported *in vivo* lipid peroxidation induced by paracetamol—its relation to hepatotoxicity. *Res Commun Chem Pathol Pharmacol* **51**: 89–99, 1986.
21. Sutton HC and Winterbourne CC, Chelated iron-catalyzed $^{\cdot}OH$ formation from paraquat radicals and H_2O_2; mechanism of formate oxidation. *Arch Biochem Biophys* **235**: 106–115, 1984.
22. Winterbourne C 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.
23. Thomas CE and Aust SD, Reductive release of iron from ferritin by cation free radicals of paraquat and other bipyridyls. *J Biol Chem* **261**: 13064–13070, 1986.
24. 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**: 366–377, 1984.
25. Aust SD, Morehouse LA and Thomas CE, Role of metals in oxygen radical reactions. *J Free Radic Biol Med* **1**: 3–25, 1985.
26. 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.

27. Beloqui O and Cederbaum AI, Prevention of microsomal production of hydroxyl radicals, but not lipid peroxidation, by the glutathione–glutathione peroxidase system. *Biochem Pharmacol* **35**: 2663–2669, 1986.
28. Puntarulo S and Cederbaum AI, Comparison of the ability of ferric complexes to catalyze microsomal chemiluminescence, lipid peroxidation and hydroxyl radical generation. *Arch Biochem Biophys* **264**: 482–491, 1988.
29. Buege JA and Aust SD, Microsomal lipid peroxidation. In: *Methods in Enzymology* (Eds. Fleischer S and Packer L), Vol. 52, pp. 302–310. Academic Press, San Diego, 1978.
30. Wills ED, Lipid peroxidation formation in microsomes: relationship of hydroxylation to lipid peroxidation formation. *Biochem J* **113**: 315–324, 1969.
31. Cederbaum AI and Cohen G, Ethanol oxidation and microsomal radical production. *Methods Enzymol* **105**: 516–522, 1984.
32. Howes RM and Steele RH, Microsomal chemiluminescence induced by NADPH and its relation to aryl-hydroxylations. *Res Commun Chem Pathol Pharmacol* **3**: 349–357, 1972.
33. Sugioka K and Nakano M, A possible mechanism of the generation of singlet molecular oxygen in NADPH-dependent microsomal lipid peroxidation. *Biochim Biophys Acta* **423**: 206–216, 1976.
34. Wright JR, Rumbaugh RC, Colby HD and Miles PR, The relationship between chemiluminescence and lipid peroxidation in rat hepatic microsomes. *Arch Biochem Biophys* **192**: 344–351, 1979.
35. Cadenas E, Boveris A and Chance B, In: *Free Radicals in Biology* (Ed. Pryor WA), Vol. 6, pp. 211–242. Academic Press, New York, 1984.
36. Goddard JG, Basford D and Sweeney GD, Lipid peroxidation stimulated by iron nitrilotriacetate in rat liver. *Biochem Pharmacol* **35**: 2381–2387, 1986.
37. Martell AE and Smith RM, *Critical Stability Constants*, Vol. 1. Plenum Press, New York, 1974.
38. Rosen H and Klebanoff SJ, Role of iron and EDTA in the bactericidal activity of a superoxide anion generating system. *Arch Biochem Biophys* **208**: 512–519, 1981.
39. Timberlake CF, Iron-malate and iron-citrate complexes. *J Chem Soc (Lond)* 5078–5085, 1964.
40. Aisen P, Some physicochemical aspects of iron metabolism. *Ciba Found Symp* **51**: 1–19, 1977.
41. Goucher CR and Taylor JF, Compounds of ferric iron with adenosine triphosphate and other nucleoside phosphates. *J Biol Chem* **239**: 2251–2255, 1964.
42. 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.
43. Osheroff MR, Schaich KM, Drew RT and Borg DC, Failure of desferrioxamine to modify the toxicity of paraquat in rats. *J Free Radic Biol Med* **1**: 71–82, 1985.
44. Cadenas E, Brigelius R and Sies H, Paraquat induced chemiluminescence of microsomal fractions. *Biochem Pharmacol* **32**: 147–150, 1983.
45. Campbell AK, Hallett MB and Weeks I, Chemiluminescence as an analytical tool in cell biology and medicine. *Methods Biochem Anal* **31**: 317–416, 1985.
46. Boveris A, Cadenas E and Chance B, Ultraweak chemiluminescence: a sensitive assay for oxidative radical reactions. *Fed Proc* **40**: 195–198, 1981.
47. Cadenas E, Biological chemiluminescence. *Photochem Photobiol* **40**: 823–830, 1984.