Biochemical Pharmacology, Vol. 31, No. 23, pp. 3723–3729, 1982.
Printed in Great Britain.

0006-2952/82/233723-07 $03.00/0

MECHANISMS OF OXYGEN ACTIVATION BY NITROFURANTOIN AND RELEVANCE TO ITS TOXICITY

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(*Received* 22 *January* 1982; *accepted* 26 *April* 1982)

Abstract—Purified ferredoxin–(cytochrome *c*)–$NADP^+$ oxidoreductase and xanthine oxidase were found to catalyse the reduction of nitrofurantoin to the free radical. Under aerobic conditions, the nitrofurantoin radical underwent autoxidation to regenerate the parent compound with the concomitant production of superoxide and eventually hydrogen peroxide. The nitrofurantoin radical was also shown to react with hydrogen peroxide to generate a highly reactive species which was capable of oxidising methionine to ethylene. This active oxygen radical appeared to be identical with the crypto-OH· radical, previously proposed as being formed from the analogous reaction of the methyl viologen radical with hydrogen peroxide [R. J. Youngman and E. F. Elstner, *FEBS Lett.* **129**, 265 (1981)]. Catalase inhibited nitrofurantoin-dependent ethylene formation in both enzyme systems, whereas superoxide dismutase was only inhibitory in the xanthine oxidase mediated reaction. Although the primary function of the respective enzyme systems is to generate the nitrofurantion radical, the xanthine oxidase reaction is markedly more complex than that of ferredoxin–(cytochrome *c*)–$NADP^+$ oxidoreductase. The differences between the two enzyme reactions appear to be due to the endogenous autoxidation of xanthine oxidase. The aerobic activation of nitrofurantoin by xanthine oxidase involved the superoxide anion as an intermediate, whereas the nitrofuran was directly reduced by ferredoxin–(cytochrome *c*)–$NADP^+$ oxidoreductase without a requirement for active oxygen species.

Nitrofurantoin [*N*-(5-nitro-2-furfurylidine)-1-aminohydantoin] (NF)* is a widely used antibacterial agent and has been shown to be particularly efficacious in the treatment of acute urinary tract infections [1–3]. Evidence has been presented that, in contrast to most other nitrofuran derivatives, NF may not be a carcinogen (see Ref. 3). The carcinogenicity of the other compounds has been suggested as being derived from the reduction of the nitro group to an active hydroxylamine [4]. Mason and Holtzman [5] demonstrated that the ESR signal due to the NF· anion radical was only detected following anaerobic reduction of the parent compound. They proposed that the lack of an ESR spectrum after aerobic incubation was due to the autoxidation of the NF· radical with the concomitant production of superoxide. Thus, under aerobic conditions, reduction of NF to the corresponding hydroxylamine would be avoided, which might explain the non-carcinogenicity of the compound.

Although NF may not possess carcinogenic properties, prolonged treatment with the drug has frequently been found to result in pulmonary fibrosis, reminiscent of paraquat toxicity. Studies into the mechanism of the pulmonary toxicity of paraquat indicated that active oxygen species are involved [6] and earlier reports suggested that NF might also be able to reductively activate oxygen [5]. Other nitrofuran derivatives have also been shown to possess similar activity [7–9]. Previous investigations have shown that microsomal preparations were able to reduce NF [11], and an increase in covalent binding of activated NF to tissue macromolecules was observed [2]. Microsomal reduction of structurally similar 5-nitrothiophenes has also been shown to occur via NADPH-dependent reaction [11]. Using partially purified preparations of NADPH–cytochrome *c* reductase and xanthine oxidase, Wang *et al.* [4] demonstrated the reduction of a range of nitrofuran derivatives including NF. Thus, although these earlier investigations indicate that microsomal NADPH–cytochrome *c* reductase and xanthine oxidase may possess nitroreductase activity, definitive evidence with purified enzymes is lacking. It was with a view to rectifying this deficiency that the studies reported here were undertaken. It was also hoped that an investigation into the oxygen-activating ability of NF would provide a further insight into the mechanism of its toxicity.

*Abbreviations: NF, nitrofurantoin [*N*-(5-nitro-2-furfurylidine)-1-aminohydantoin]; MV, methyl viologen [paraquat (1,1′-dimethyl-4,4-bipyridylium dichloride)]; AQ, anthraquinone-2-sulphonate; Fd–(cyt. *c*)–NADP-OR, ferredoxin–(cytochrome *c*)–$NADP^+$-oxidoreductase; SOD, superoxide dismutase; EtOH, ethanol.

MATERIALS AND METHODS

Enzymes and chemicals. Fd–(cyt. *c*)–NADP-OR was isolated from *Euglena gracilis* and purified according to Lengfelder and Elstner [12]. This preparation possessed a single enzyme activity and had previously been shown to couple with ferredoxin, anthraquinone [13] or paraquat [14] as the electron acceptor. The basic reaction of the Fd–(cyt. *c*)–NADP-OR system as used in the subsequent experiments has already been described [14]. SOD was isolated from dried green peas as described in Ref. 15.

Table 1. Oxygen uptake mediated by Fd–(cyt. *c*)–NADP-OR

Addition	Oxygen uptake (μmoles of O_2/hr)	
	− catalase	+ catalase (100 U)
None	0.1	0.1
NF (5×10^{-5} M)	0.3	0.1
NF (5×10^{-4} M)	3.4	1.8
NF (2.5×10^{-3} M)	9.7	5.7
AQ (5×10^{-5} M)	3.9	1.9
AQ (5×10^{-4} M)	17.2	9.3
MV (5×10^{-5} M)	0.4	0.2
MV (5×10^{-4} M)	2.6	1.1

Reaction conditions; the reaction mixture contained in 2 ml: 10 μmoles of glucose-6-phosphate; 50 μg of glucose-6-phosphate dehydrogenase; 1 μmole of NADP; Fd–(cyt. *c*)–NADP-OR containing 0.1 mg of protein; 200 μmoles phosphate buffer (pH 7.8); electron acceptor concentrations as indicated.

Catalase, glucose-6-phosphate dehydrogenase and xanthine oxidase were obtained from Boehringer (Mannheim, West Germany).

Assays. Oxygen exchange in the reaction mixture was monitored using a Hansatech oxygen electrode (Bachofer, Reutlingen, F.R.G.). Superoxide was determined by its ability to oxidise hydroxylamine to nitrite as already described [16] with the following modifications: sulphanilic acid was replaced by 1% sulphanilamide in 25% HCl, α-naphthylamine was replaced by 0.02% naphthylethylene diamine diHCl and the absorption of the colour reaction was measured at 540 nm.

Crypto-OH· radical formation was detected by its ability to form ethylene from methionine in the presence of pyridoxal phosphate under the conditions described previously [13, 14]. The reactions were conducted in Fernbach flasks sealed with serum rubber stoppers. Ethylene was determined by gas chromatography [17]. Anaerobic conditions were achieved and maintained as in Ref. 14.

RESULTS

The Fd–(cyt. *c*)–NADP-OR used in the present studies was found to reduce both horse heart cytochrome *c* and cytochrome c_{552} from *E. gracilis* in a NADPH-dependent reaction (manuscript in preparation). Thus the enzyme possessed an identical activity to NADPH–cytochrome *c* reductase preparations used in previous studies [2, 4, 5, 10].

Oxygen exchange

Table 1 shows that NF was reduced via Fd–(cyt. *c*)–NADP-OR and underwent autoxidation, thereby resulting in a stimulation of oxygen uptake which was dependent upon the NF concentration. The observed rate of oxygen uptake was similar to that obtained with paraquat, but was much lower than that with anthraquinone as the electron acceptor. The addition of catalase to the reaction mixture resulted in a 50% decrease in the rate of oxygen uptake with all three acceptors, indicating that H_2O_2 was a product of the autoxidation reactions.

In contrast, NF stimulated oxygen uptake by only up to about 30% in xanthine oxidase dependent reaction (Table 2). This enzyme system is able to reduce dioxygen directly to O_2^- and H_2O_2 without the requirement of an added electron mediator. The addition of oxygen uptake, which, although less than in the Fd–(cyt. *c*)–NADP-OR reaction, again indicated the presence of H_2O_2.

Superoxide formation

The formation of superoxide was strongly stimulated by the presence of NF in both enzymatic systems (Fig. 1a and b). The addition of 20 U SOD to reactions containing 0.5 mM NF resulted in inhibition, thus confirming that superoxide was responsible for the stimulation of nitrite production and not some other reduced NF intermediate. These results are consistent with the data of Mason and Holtzman [5], who postulated that superoxide was the first intermediate formed during the autoxidation of NF·.

Table 2. Oxygen uptake mediated by xanthine oxidase

Addition	Oxygen uptake (μmoles/hr)
None	0.68
NF (5×10^{-4} M)	0.70
NF (1×10^{-3} M)	0.88
NF (1×10^{-3} M) + 100 U catalase	0.53

Reaction conditions: reaction mixture contained in 2 ml: 1 μmole of xanthine; 40 μg of xanthine oxidase; 200 μmoles of phosphate buffer (pH 6.0); other additions as indicated.

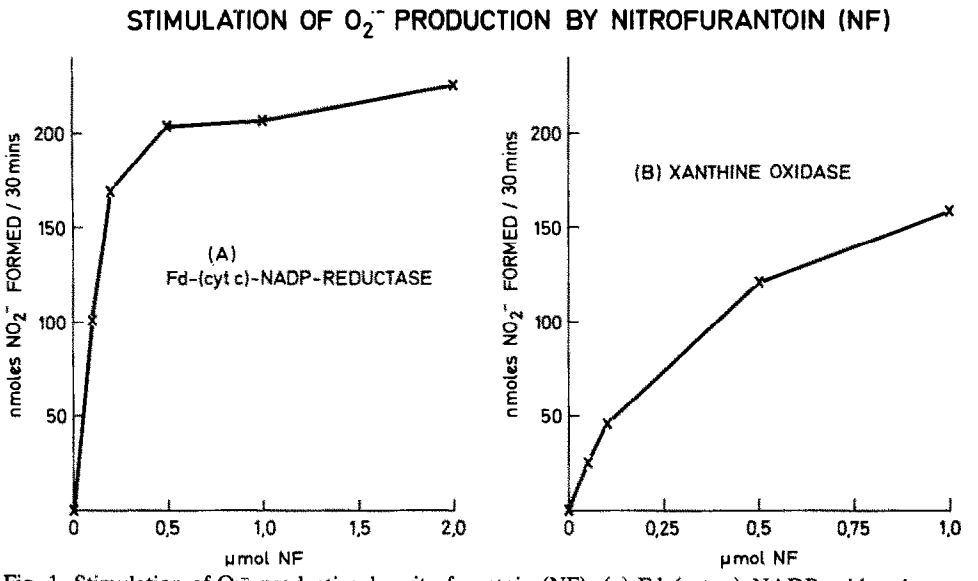

Fig. 1. Stimulation of $O_2^{\cdot-}$ production by nitrofurantoin (NF). (a) Fd–(cyt. *c*)–NADP-oxidoreductase system; reaction conditions as described in Table 1 with the addition of 1 μmole of NH_2OH. At the end of the reaction, NO_2^- was determined. (b) Xanthine oxidase system; details as given in Table 2 with the addition of 1 μmole of NH_2OH. NO_2^- was determined. The background level of NO_2^- generated by the action of xanthine oxidase alone was subtracted from all values. This was not necessary for the Fd–(cyt. *c*)–NADP-oxideroductase system which was not autoxidisable.

Crypto-OH· formation

The formation of ethylene from methionine and pyridoxal phosphate has been shown to be dependent upon the presence of pulse radiolytically produced *free* OH· radicals [18], but not by $O_2^{\cdot-}$, H_2O_2 or both in combination, nor by the xanthine oxidase reaction alone [13, 14, 19]. The formation of ethylene from the methionine analogue methional is not a specific test for OH· radicals [20]. In previous studies, it was found that although ethylene was produced from methional and 2-keto-4-mercaptomethylbutyrate via both peroxidase-H_2O_2 [21] and chloroplast lamellae dependent reactions [22], methionine alone was inactive as a substrate.

Under the conditions described here, the species responsible is the crypto-OH· radical which is formed

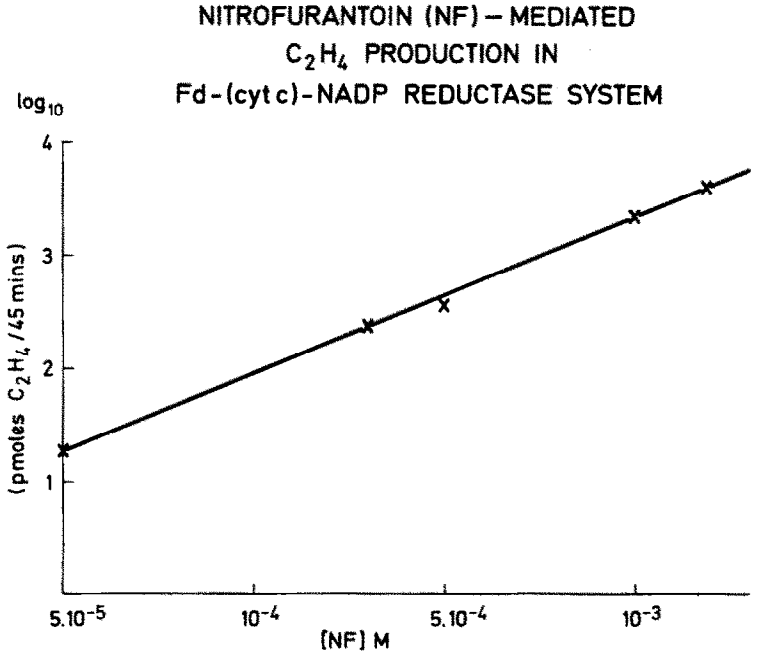

Fig. 2. Nitrofurantoin (NF)-mediated C_2H_4 production in Fd–(cyt. *c*)–NADP-oxidoreductase system. Reaction conditions were as described in Table 1 with the addition of 20 μmoles of methionine and 0.2 μmoles of pyridoxal phosphate. The ~10-ml Fernbach flasks were incubated at 22° for 45 min, followed by GLC determination of ethylene.

Table 3. NF-mediated ethylene formation

Additions	Ethylene formation	
	Fd–(cyt.*c*)–NADP-OR (pmoles/45 min)	Xanthine oxidase (pmoles/90 min)
None	5	53
AQ (10^{-4} M)	1644	114
MV (10^{-4} M)	12	—
NF (5×10^{-4} M)	203	251
NF + SOD	206 (200 U)	13 (60 U)
NF + 100 U catalase	19	35

Reaction conditions as described in legends to Tables 1 and 2 with the addition of 20 μmoles methionine, and 0.2 μmoles pyridoxal phosphate. The reactions were conducted in 10-ml Fernbach flasks at 22° for the times indicated.

by electron donation to H_2O_2 [13, 14]. In order for a compound to give rise to this active oxygen species, it should be capable of being univalently reduced to a radical which is then able to react with H_2O_2.

Ethylene formation in an Fd–(cyt. *c*)–NADP-OR-catalysed reaction is shown in Fig. 2. NF clearly stimulated the production of this hydrocarbon in a concentration-dependent manner. This increase was only about 10% of that observed with anthraquinone as the electron acceptor, but was significantly greater than that obtained with paraquat (Table 3). Catalase completely inhibited NF-enhanced ethylene formation, whereas SOD was without effect (Table 3). The presence of traditional free OH· radical scavengers stimulated ethylene production by up to 50% (in the case of mannitol) when present at a concentration of 1 mM (Table 4). Increasing the concentration 10-fold resulted in an inhibition of ethylene formation of up to about 50% (with benzoate). The other non-specific free radical scavenger, *n*-propyl gallate, completely inhibited the reaction at a concentration of 100 μM. It should be noted that the inhibition by free OH· scavengers at high concentrations (10 mM) was inversely proportional to the respective stimulations observed at lower levels (1 mM) of the compounds.

In previous studies, paraquat was shown to be ineffective in catalysing ethylene formation under aerobic conditions [14], but the hydrocarbon was detected when the oxygen level in the reaction flasks was decreased. Large amounts of ethylene were formed under strict anaerobiosis in the presence of H_2O_2, which was taken as strong evidence of a reaction between reduced paraquat and H_2O_2, resulting in the production of the crypto-OH· radical [14]. Table 5 shows the results of a similar experiment with NF as the electron acceptor. In this reaction, NF was able to strongly stimulate ethylene formation. Under strict anaerobiosis in the absence of H_2O_2, virtually no ethylene was formed. The presence of a low level of oxygen (~1 μmole) in the reaction flask permitted the formation of ethylene with both NF and paraquat as the electron acceptor. Normal atmospheric levels of oxygen completely inhibited ethylene formation in the presence of paraquat [14], but hydrocarbon production was only reduced by 50% with NF as the electron acceptor as compared to levels obtained under partial anaerobiosis.

NF also stimulated ethylene production from methionine and pyridoxal phosphate in the xanthine oxidase system (Table 3). Catalase was a potent inhibitor of this reaction as was SOD, in contrast to the Fd–(cyt. *c*)–NADP-OR system. Various free OH· radical scavengers slightly inhibited or stimulated ethylene formation at low concentrations which

Table 4. Effect of scavengers on ethylene formation

Addition	Ethylene formation (pmoles/45 min)	
	Fd–(cyt.*c*)–NADP-OR	Xanthine oxidase
NF (5×10^{-4} M)	264	190
NF + EtOH (10^{-3} M)	319	146
NF + benzoate (10^{-3} M)	303	175
NF + mannitol (10^{-3} M)	397	210
NF + *n*-propyl gallate (10^{-4} M)	9	15
NF	360	190
NF + EtOH (10^{-2} M)	230	95
NF + benzoate (10^{-2} M)	179	68
NF + mannitol (10^{-2} M)	269	124

Reaction conditions as described in legend to Table 3.

Table 5. Oxygen of NF-mediated ethylene formation

Conditions	Ethylene formation (pmoles/45 min) − NF	+ NF (5×10^{-4} M)
Anaerobiosis	0	5
Partial anaerobiosis (~ 1 μmoles O_2/flask)	4	2650
Aerobiosis (~ 100 μmoles of O_2/flask)	6	1300
Anaerobiosis + 10 μmoles of H_2O_2	168	23,190

Reaction conditions as described in Table 3 with Fd–(cyt. *c*)–NADP-OR).

gave way to moderate inhibition at higher levels (Table 4). Under anaerobic conditions, NF reduced by xanthine oxidase reacted with exogenously supplied H_2O_2 to form a species which was able to oxidise methionine to ethylene [23]. This is further evidence that in the presence of H_2O_2 the sole function of the respective enzyme system is to provide a source of reduced NF.

DISCUSSION

The studies reported here demonstrate that the widely used drug NF can be readily reduced by pure preparations of both Fd–(cyt. *c*)–NADP-OR and xanthine oxidase. The Fd–(cyt. *c*)–NADP-OR used in these investigations was found to possess the same activity as microsomal NADPH–cytochrome *c* reductase and thus the results presented here can be directly compared with the earlier data in Refs 2, 4, 5 and 10. In agreement with these previous studies, NF can be reduced to a free radical which autoxidises in the presence of dioxygen to regenerate the parent nitrofuran derivative. Mason and Holtzmann [5] showed that an ESR signal could be detected following incubation of NF with microsomes and found that this was due to the NF· radical. Reduction of NF via the FD–(cyt. *c*)–NADP-OR system under anaerobic conditions gave an ESR spectrum (data not shown), similar to that published by Mason and Holtzmann [5] and thus confirmed NF· production in the pure enzymatic system.

There have been previous indications that NF reduction may be catalysed by NADPH–cytochrome *c* reductase and xanthine oxidase [4]. The present study confirms this proposal and also discriminates between reduction of NF by xanthine oxidase and Fd–(cyt. *c*)–NADP-OR. In both enzymatic reactions, NF was able to catalyse or stimulate ethylene formation from methionine via the production of a strong oxidant (crypto-OH· radical [14]). Although the production of this reactive species need not directly involve O_2^-, as was shown by the Fd–(cyt. *c*)–NADP-OR system, the presence of SOD in the xanthine oxidase reaction resulted in a total inhibition of ethylene formation. This is an indication of the differing complexities of the two enzymatic systems. In the Fd–(cyt. *c*)–NADP-OR reaction, NF is directly reduced by the enzyme and oxygen activation occurs solely as a result of NF· autoxidation.

In order to comprehend the observed effects with xanthine oxidase, the endogenous mechanism of this electron transport system must be considered. The enzyme system is known to consist of a sequence of electron carriers which interact with dioxygen to produce H_2O_2 and O_2^- [24]. The ratio between the levels of these two reduced oxygen species varies with the age of the enzyme and thus it would appear that they are produced independently of one another. The relative stimulation of O_2^- production by NF was considerably larger than the enhancement of oxygen uptake and H_2O_2 formation, which suggests that the nitrofuran interacted mainly with the pathway leading to O_2^- generation. It is envisaged that NF overcame a rate-limiting step in the endogenous univalent reduction of dioxygen and thus resulted in an increase in O_2^- production. In order to react in such a manner, NF would have to approach the enzyme very closely, probably entering a cavity near the active site(s) of the enzyme. It is therefore important to distinguish between electron transfer reactions occurring close to the enzyme and those which probably take place some distance away.

Reactions occurring at or very close to xanthine oxidase:

$$NF + e^- \rightarrow NF\cdot \quad (1)$$

$$O_2 + e^- \rightarrow O_2^- \quad (2)$$

$$NF\cdot + O_2 \rightleftarrows NF + O_2^- \quad (3)$$

$$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2 \quad (4)$$

$$NF\cdot + H_2O_2 \rightarrow (NF\cdot\text{–}H_2O_2) \quad (5)$$
$$\text{crypto-OH}\cdot$$

Reactions occurring some distance away from xanthine oxidase:

$$NF + O_2^- \rightleftarrows NF\cdot + O_2 \quad (6)$$

$$2O_2^- + H_2^+ \rightarrow H_2O_2 + O_2 \quad (7)$$

$$NF\cdot + H_2O_2 \rightarrow (NF\cdot\text{–}H_2O_2) \quad (8)$$
$$\text{crypto-OH}\cdot$$

This reaction sequence is dependent on the ability of O_2^- to diffuse away from the immediate vicinity of the enzyme to the general reaction milieu where the NF/NF· ratio would be greater than closer to the enzyme. The reaction between NF and O_2^- is

reversible and near the enzyme the equilibrium would be expected to favour the production of O_2^- [reaction (3)]. Further away from xanthine oxidase, the equilibrium would be reversed and thus NF· production would be increased [reaction (6)]. This could then react with H_2O_2, produced directly via xanthine oxidase or from the dismutation of O_2^-, to form the crypto-OH· radical. It is proposed that only crypto-OH· radicals formed some distance away from xanthine oxidase are able to oxidise methionine to ethylene. Since the generation of NF· required for the production of this species is dependent on O_2^-, SOD was able to effectively inhibit ethylene formation. The observation that O_2^- formation was increased by NF can be explained by supposing that O_2^- reacted preferentially with hydroxylamine (forming nitrite) instead of with NF.

Although NF mediated ethylene formation under aerobic conditions, the reaction was markedly stimulated by reducing the oxygen tension in the reaction vessel. The inhibition by catalase and the lack of a SOD effect in the Fd–(cyt. *c*)–NADP-OR system was reminiscent of MV reactions [14] and suggested the formation of crypto-OH· radical from NF· and H_2O_2. Further evidence was obtained from experiments under complete anaerobiosis. In the presence of added H_2O_2 under these conditions, NF was able to form ethylene in a similar manner to MV [14]. Thus, NF appeared to activate oxygen via an MV-type mechanism, although the observation that ethylene was also formed in air suggests that the NF· radical was somewhat more stable than that of MV under aerobic conditions, and thus some crypto-OH· radical production was still able to occur (cf. AQ in Table 3).

The effects of traditional free OH· scavengers were dependent upon the concentration and differences were most apparent in the Fd–(cyt. *c*)–NADP-OR system. The stimulation of ethylene production by low scavenger concentrations (1 mM) may be explained in terms of a 'cage effect' [25, 26], whereby NF· and H_2O_2 react to form a crypto-OH· radical within a cage of solvent molecules. It is possible that a small proportion of free OH· radicals may be released from the crypto-OH· complex and escape from the cage. In the absence of OH· scavengers these radicals destroyed the enzyme(s) and thus led to decreased activity [27]. However, in the presence of low scavenger concentrations, OH· escaping from the cage was prevented from causing enzyme destruction and thus increased ethylene production was observed. At higher scavenger concentrations (10 mM), competition existed between the scavenger and methionine for crypto-OH· radicals and thus an inhibition of the formation of ethylene occurred.

From these studies, it is clear that NF was readily reduced by two ubiquitous enzymatic systems, both of which are known to be closely involved in oxygen activation. NF was able to reductively activate oxygen in a similar manner to AQ and MV, which led to the production of a strong oxidant, the crypto-OH· radical. The mechanism of formation of this species was also comparable with the other systems, namely resulting from electron donation from NF· to H_2O_2 and not via a catalysed Haber–Weiss reaction.

Possible mechanisms of NF toxicity

It has been reported that the pulmonary toxicity of NF is manifested by damage similar to that induced by paraquat (MV) [1]. The present communication provides evidence that both NF and MV can reductively activate oxygen via similar mechanisms in *in vitro* systems. Both of these compounds were able to produce the highly reactive crypto-OH· radical which might account for their *in vivo* toxicity. The free radical of NF appears from our results to be more stable in the presence of oxygen as compared to $MV^{\cdot+}$ and thus it may be supposed that tissue oxygen concentrations are less critical in order for NF to exert a toxic effect via this mechanism.

A reaction between $MV^{\cdot+}$ and O_2^- to form a highly reactive adduct has been demonstrated by Nanni *et al.* [28]. However, the lack of an effect by SOD in the Fd–(cyt. *c*)–NADP-OR-dependent methionine oxidation tends to rule out the formation of a similar species from the reaction of NF· and O_2^-. Our results indicating the formation of crypto-OH· radicals from an electron donor and H_2O_2 agree closely with a report by Levey *et al.* [26] of a reaction between $MV^{\cdot+}$ and H_2O_2 in a pure chemical system. The product in these studies was not the free OH· radical, as high concentrations of methanol were without effect.

In summary, the formation of crypto-OH· radicals by NF may play a central role in the mechanism of the toxicity of the compound. The production of the two radical-generating species, NF· and H_2O_2, could be readily achieved by NADPH–cytochrome *c* reductase in the lung.

Acknowledgements—The authors are grateful to Dr V. Breunninger for the cooperation in verifying the formation of the NF radical by the use of ESR techniques. This work was supported by the Deutsche Forschungsgemeinschaft.

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