Biochemical Pharmacology, Vol. 25, pp. 393–398. Pergamon Press, 1976. Printed in Great Britain.

ELECTRON TRANSFER IN EHRLICH ASCITES TUMOR CELLS IN THE PRESENCE OF NITROFURANS*

JOHN E. BIAGLOW, ODDVAR F. NYGAARD and CLIVE L. GREENSTOCK

Division of Radiation Biology, Department of Radiology, Case Western Reserve University, Cleveland, Ohio, 44106 U.S.A., and Medical Biophysics Branch, Whiteshell Nuclear Research Establishment, Atomic Energy of Canada Limited, Pinawa, Manitoba R0E 1L0, Canada

(*Received* 24 *February* 1975; *accepted* 23 *May* 1975)

Abstract –Electron-affinic nitrofuran derivatives interfere with normal cellular metabolism by providing an electron shunt, apparently via free radical intermediates, between endogenous cellular reducing species and oxygen, in a manner analogous to that of vitamin K_3. Pulse radiolysis was used to demonstrate the reactivity of nitrofuran radical anions with oxygen, as well as the NAD free radicals with nitrofurans. The reduction of nitrofurans under anaerobic conditions and the increased oxygen consumption (indicative of free radical formation) are enhanced by the addition of glucose and suppressed by the removal of endogenous reducing species, e.g. by the addition of diamide. Nitrofuran free radical production under aerobic conditions may result in the production of the superoxide radical anion O_2^-. It is postulated that aerobic production of nitrofuran or oxygen free radicals or the resulting products may be responsible for the previously described cytotoxic effect of nitrofurans.

Nitrofuran (NF) derivatives have found a new application as anoxic radiation sensitizers of mammalian cells. Radiation chemical and radiobiological studies show that in this use of compounds the electron affinity of nitrofuran derivatives is an important chemical property which determines the extent of their interaction with radicals formed in DNA by radiation [1, 2]. While the ability to accept electrons makes this class of chemicals potentially valuable tools for studying mechanisms of radical involvement in cellular radiation damage, it may also be the reason why nitrofurans have been found to be radiomimetic, mutagenic and carcinogenic [3, 4] and cytotoxic [5], as well as inhibitors of electron transfer reactions [6, 7]. Presumably, some of these effects are caused by the products of reductive metabolism of the drugs. However, the metabolic reduction of the drug has only been demonstrated under anaerobic conditions [8], while most of the cellular effects listed above occur in the presence of oxygen [9]. This paper will deal with the factors involved in the metabolism of the nitrofurans under aerobic and metabolically produced anaerobic conditions. The reaction of NF with coenzyme free radicals (presumed metabolic intermediates) and reactivity of NF free radicals with molecular oxygen will be reported.

EXPERIMENTAL

Ehrlich ascites tumor (EAT) cells were grown in mice and harvested as previously described [10]. Oxygen measurements were made with the aid of a Clark oxygen electrode apparatus (Yellow Springs Instrument Co.). Fluorescence measurements were made with either the Bowman–Aminco or the Eppendorf spectrofluorometer. Absorption spectra were obtained with the aid of a Cary model 15 spectrophotometer, fitted with an automatic cell changer. Absorption spectra on dense cell suspensions were determined with the use of the Cary micrometer screw cuvette.

The nsec pulse radiolysis data of direct measurements of redox reaction rates were obtained using single 50-nsec pulses from the 3-MeV Van de Graaff generator at the Whiteshell Nuclear Research Establishment. The average dose per pulse to the sample was 0·2 to 2 krad. The associated optical and electronic apparatus is similar to that described previously [11]. Solutions were saturated with the appropriate gas, with the use of a multisyringe bubbling technique, and the irradiation cell was filled and back-flushed between experiments by a remote-control flow system [12]. Oscilloscopic traces of the transient species detected by kinetic spectrophotometry were digitized and the bimolecular rate constants for the redox reactions determined by computer.

The biochemicals (Koch-Light Lab. Ltd., Sigma Chemical Co.) and the nitrofuran derivatives, nifuroxime and nitrofurazone (Pfaltz & Bauer, Aldrich Chemical Co.), were used without further purification in triply distilled water.†

RESULTS AND DISCUSSION

Metabolic reduction of nitrofurans. The reduction of NF is known to occur under anaerobic conditions [8]. However, the procedure for measuring the reduced drugs is rather tedious. We have found it convenient to measure the change in the characteristic absorption spectrum of one of the nitrofurans, nitrofurazone, in a dense cell suspension made anaerobic

* This investigation was supported by Public Health Research Grant No. CA-13747 from the National Cancer Institute.

† The two different nitrofuran derivatives were used in the present work because the absorption spectra of the compounds and their reduction products offered specific advantages in different systems; however, in each case where both compounds have been tested, their effects were qualtitatively the same and quantitatively very similar.

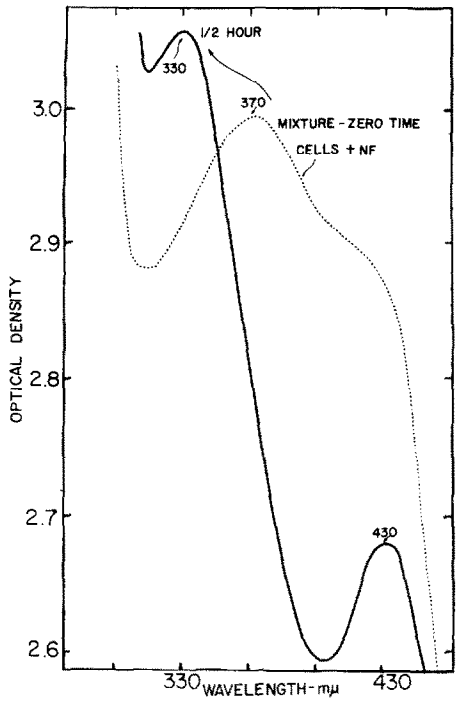

Fig. 1. Change in absorption spectrum of nitrofurazone in the presence of EAT cells. The spectrum of nitrofurazone shifts upon reduction from 370 to 330 nm. The cells (2×10^8/cm^3) were suspended in 0·05 M phosphate buffered saline, pH 7·0, 25°. The nitrofurazone was $2{\cdot}5 \times 10^{-4}$ M.

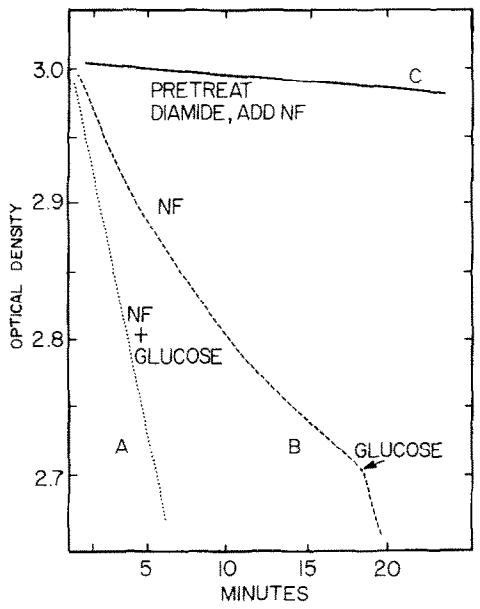

Fig. 2. Reduction of nitrofurazone by dense suspensions of EAT cells. The conditions are the same as those listed in the legend of Fig. 1. Curve A shows the reduction of 250 μM nitrofurazone in the presence of 0·01 M glucose. Curve B, initial portion, shows the reduction of nitrofurazone in the absence of glucose; 10 μmoles glucose was added where indicated. Curve C (top) shows the effect of pretreating the cells with 50 μmoles diamide in the absence of glucose. (measurements at 370 nm).

by cellular consumption of oxygen. The method was further facilitated by the use of the micrometer screw cell with a 0·05-mm light path for recording the kinetics of drug reduction. The absorption spectrum of the cell suspension (2×10^8 cells/ml) with drug was determined before incubation, as is shown in Fig. 1. After 0·5 hr of incubation at 25°, a new absorption spectrum was obtained, indicative of the metabolically reduced drug. Dithionite-reduced nitrofurazone produced the same absorption spectrum. Oxygen uptake measurements at this cell density indicate that the rate of consumption was sufficient to maintain the cell suspension at extreme hypoxia in the optical portion of the micrometer cuvette.

The reduction of the drug obviously requires cellular reducing equivalents, possibly mediated by NADPH [13], as demonstrated for the microsomal reduction of nitrofurans [14]. Removal of this intracellular reducing capacity should decrease the rate of reduction of the drug. Diamide, a sulfhydryl oxidizing agent, can remove reducing equivalents from whole cells [10]. For this reason it was of interest to determine the influence of prior removal of cellular reducing capacity on the rate of reduction of nitrofurazone. Figure 2 shows the decrease in the reduction rate that is obtained if cells are pretreated with diamide (solid line) or the increase in the rate of reduction if the pool of reducing equivalents is increased by the addition of glucose (dotted line). The dashed line shows that the rate of reduction decreases upon an apparent exhaustion of intracellular reducing equivalents but is again stimulated by the addition of glucose. No accumulation of reduced nitrofurazone was observed in the presence of oxygen.

Effect of nitrofurans on O_2 consumption. The failure to detect a metabolically reduced nitrofuran derivative in the presence of oxygen may indicate either that cellular metabolism under aerobic conditions is not capable of reducing the drug or that the reduced intermediates are rapidly re-oxidized. A biochemical means for determining whether nitrofurans cause altered cellular electron transfer is to examine their effect on the rate of oxygen consumption by intact cells. As seen in Fig. 3, curve A, aerobic EAT cells in phosphate buffer with glucose present show an immediate stimulation of oxygen consumption upon NF addition that continues uninterrupted for several min until either O_2 or substrate is depleted from the medium. NF addition to cells consuming oxygen in the absence of glucose causes an initial stimulation of oxygen consumption followed by a progressive inhibition, which may result from either depletion of reducing equivalents or a gradual inhibition of the respiratory enzymes (curve B). If diamide is added to remove reducing equivalents from cells in glucose-free medium, there is a pronounced inhibition of oxygen utilization when NF is added (curve C).

Evidence for the possible mechanism by which NF stimulated oxygen consumption was obtained by pretreating the cells with 0·0013 M KCN, an inhibitor of mitochondrial oxidations. As seen in Fig. 4, the addition of NF to KCN-inhibited cells in the presence

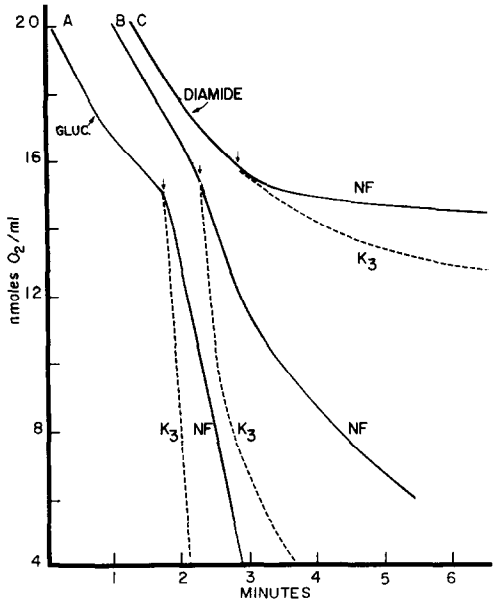

Fig. 3. Utilization of oxygen by EAT cells. The effects of nifuroxime and menadione on cellular oxidation, in the presence and absence of glucose, were determined. Oxidation was initiated by the addition of the cells to the reaction medium consisting of 0·05 M phosphate buffer in physiological saline, pH 7·0, 37°. The concentration of agents added indicated by the arrows was 10 μmoles glucose, 2·5 μmoles diamide, 5 μmoles K_3 and 5 μmoles NF.

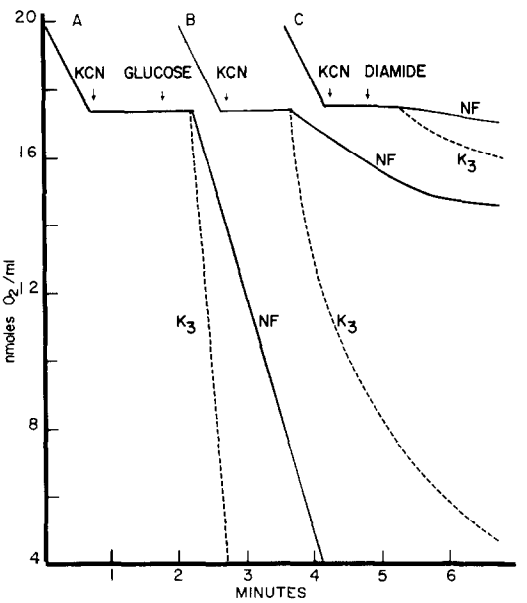

Fig. 4. Stimulation (turn-on) of oxygen utilization by nifuroxime and K_3 in KCN-inhibited EAT cells. The reaction medium is the same as that indicated in the legend of Fig. 3. The concentration of agents added indicated by the arrows was 4 μmoles KCN, 2·5 μmoles diamide, 10 μmoles glucose, 5 μmoles K_3 and 5 μmoles NF.

of glucose (curve A) results in a marked resumption of oxygen utilization, which continues until either glucose or oxygen is exhausted from the medium. NF addition to cells without glucose (curve B) also results in an initial stimulation followed by a progressive inhibition, but the onset of inhibition appears to be more rapid. Diamide pretreatment (curve C) prevents the turn-on of oxygen utilization when NF is added. Since KCN is a known inhibitor of mitochondrial cytochrome oxidase, these results suggest that the non-mitochondrial reduction product of NF might react directly with molecular oxygen.

Support for this possible mode of action of NF in aerobic cells was obtained by adding vitamin K_3 (a compound known to be able to shunt electrons directly to oxygen [15]) to EAT cells maintained under the various conditions indicated in Figs. 3 and 4. As will be seen (dashed curves), K_3 produced qualitatively the same effects as did NF, but the effects observed with K_3 were more pronounced.

Possible involvement of free radical intermediates in electron transfer with NF. As seen in Fig. 5, a solution of NF that has been reduced by dithionite consumes oxygen at a very low rate. If, however, untreated NF is added, there is a great increase in oxygen uptake. A similar, rapid uptake of oxygen can also be demonstrated with a partially reduced dithionite product of NF which has a dark maroon color, suggesting the presence of a stabilized NF free radical (charge transfer complex). The same effect can be obtained if oxidized flavin monoucleotide (FMN) is added to the reduced NF. Finally, the stabilized free radical formed by the mixing of NADH and FMN can be oxidized by NF in the absence of oxygen.

Pulse radiolysis was used to look at both the production and reactivity of nitrofuran free radicals in a simple chemical system. Following a 50-nsec pulse of high energy electrons incident on the aqueous solutions under study, roughly equal amounts of the two principal reactive species, hydrated electrons (e^-_{aq}) and hydroxyl free radicals (·OH), are formed, together with a small yield of hydrogen atoms (H·) as seen in reaction 1.

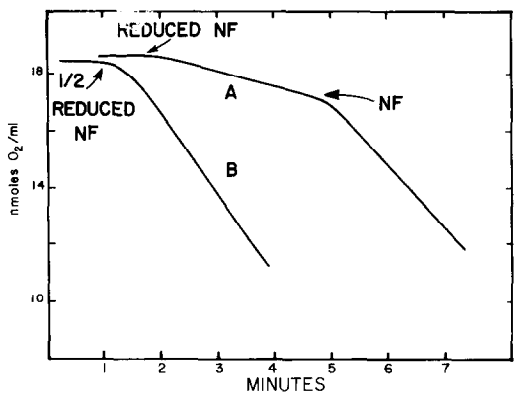

Fig. 5. Oxygen consumption by dithionite-reduced nifuroxime. The reaction medium is the same as that listed in the legend of Fig. 3. Curve A shows oxygen consumption by 9 μmoles of dithionite-reduced nifuroxime; at the arrow, 18·5 μmoles nifuroxime was added. Curve B shows oxygen consumption by 20 μmoles of half-reduced nifuroxime (maroon-colored complex).

In the presence of a suitable H· and ·OH scavenger, *t*-butanol (reaction 2), only the remaining e_{aq}^- reacts with the nitrofurans to form the nitrofuran radical anion species NF^- (reaction 3), which has a strong characteristic absorption at 390 nm [12]. In the absence of any electron acceptor, the NF^- species is quite long-lived and ultimately decays by a slow-order reaction (reaction 4). However, in the presence of excess oxygen, the NF^- absorption is found to decay with first-order kinetics, providing direct evidence for the feasibility of oxidation of metabolically produced reduced nitrofuran intermediates by oxygen (reaction 5).

$$H_2O \rightsquigarrow e_{aq}^-, {\cdot}OH, H{\cdot} \quad (1)$$

$$\left.\begin{matrix}{\cdot}OH \\ \text{or} \\ H{\cdot}\end{matrix}\right\} + (CH_3)_3COH \rightarrow$$

$$(CH_3)_2\dot{C}H_2COH + \left\{\begin{matrix}H_2O \\ \text{or} \\ H_2\end{matrix}\right. \quad (2)$$
(inert)

$$e_{aq}^- + NF \rightarrow NF^- \quad (3)$$

$$NF^- + NF^- \rightarrow \text{slow} \quad (4)$$

$$NF^- + O_2 \rightarrow \text{fast} \quad (5)$$

Figure 6 shows semilogarithmic plots of the first-order decays of NF^- and the corresponding half-lives ($T_{\frac{1}{2}}$) at different oxygen concentrations. The absolute rate constant $k = 0{\cdot}693/[O_2]T_{\frac{1}{2}}$ for the reaction of NF^- with oxygen obtained from these data is $1{\cdot}5 \times 10^9\ M^{-1}\ sec^{-1}$. A similar result was found for the reaction of the electron adduct species of the known oxygen-shuttling molecule vitamin K_3 with oxygen (rate constant $0{\cdot}2 \times 10^9\ M^{-1}\ sec^{-1}$). Both rate constants are high, being close to the diffusion-controlled limit.

Both flavin and pyridine nucleotides may be reduced metabolically by a two-step process via free

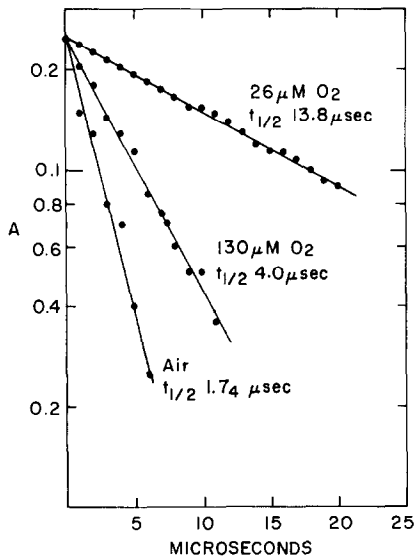

Fig. 6. Effect of oxygen concentration on the rate of decay of nifuroxime radical union. The decays, which were measured at 400 nm in deoxygenated 2 mM nifuroxime solutions containing 0·5 M *t*-butanol, are all of exponential form.

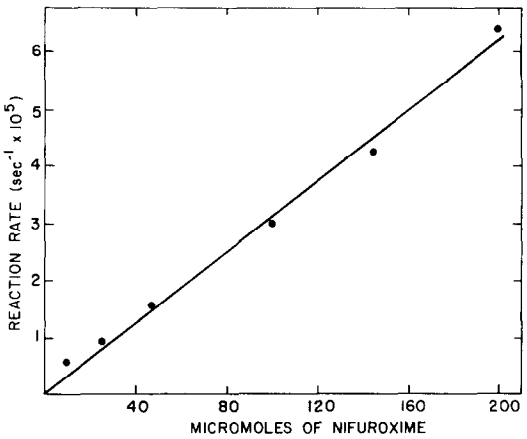

Fig. 7. Rate of oxidation of pyridine nucleotide free radicals in the presence of various concentrations of nifuroxime. The NF^- buildups, observed after the pulse, in deoxygenated 2 mM NAD solutions containing 0·5 M *t*-butanol, show a first-order dependence on nifuroxime concentration.

radical intermediates. The same free radical intermediates were produced using the pulse radiolysis technique, and their interaction with the colorless nitrofuran derivative, nifuroxime, was studied. As an example, 2 mM oxidized pyridine nucleotide (NAD^+) was reacted with e_{aq} to form pyridine nucleotide radicals (NAD·) (reaction 6). In the absence of oxygen, pyridine nucleotide radicals were found to react first order with nifuroxime with a rate constant of $3{\cdot}1 \times 10^9\ M^{-1}\ sec^{-1}$. Figure 7 shows the concomitant rate of formation of reduced NF^- as a function of nifuroxime concentration as a result of the one-electron oxidation of NAD· (reaction 7).

$$NAD^+ + e_{aq}^- \rightarrow NAD{\cdot} \quad (6)$$

$$NAD{\cdot} + NF \rightarrow NAD^+ + NF^- \quad (7)$$

Similarly, pyridine nucleotide radicals have been shown previously to be oxidized by oxygen, the rate constant for the reaction being $2{\cdot}0 \times 10^9\ M^{-1}\ sec^{-1}$ [16–18].

DISCUSSION

The ability of the nitrofuran derivatives to accept electrons makes them oxygen mimics with respect to radical scavenging activity during X-irradiation. The same property of electron acceptance causes interference with cellular electron transfer reactions through reduction of the drug and its subsequent reaction with either oxygen or cellular electron acceptors. Polarographic studies under anaerobic conditions indicate that the nitro group is capable of forming stable products by accepting either four or six electrons [19]. We have found that the dithionite-reduced product, which most likely is the hydroxylamine, has the same absorption spectrum as the metabolically reduced derivative (Fig. 1); this product is relatively stable toward oxygen until oxidized nitrofuran (or FMN) is added (Fig. 5). Free radical NF derivatives may be formed

if the anaerobically reduced product reacts with electron acceptors such as NAD^+ or FAD-FMN or with oxidized NF. Our preliminary studies in chemical systems [20] indicate that these complexes are possible (Fig. 5). That free radical intermediates produced by such an interaction are extremely reactive toward oxygen, as indicated by the pulse radiolysis data (Fig. 6).

The failure to demonstrate the formation of reduced nitrofurans with acrobically growing cells [8] may be explained if the nitrofuran free radical is an intermediate first step in its cellular reduction, similar to the free radical intermediates of flavin and pyridine nucleotides. Such an intermediate obviously would be extremely active toward oxygen. The reason that cellular NAD(P) and flavin free radicals do not react directly with oxygen or oxidizing reactants is that they may be protected through protein interaction via tryptophan residues [21]. Additional support for the direct interaction of nitrofuran free radicals with oxygen is provided by the similar behavior in aerobic and KCN-inhibited cells of K_3 (Figs. 3 and 4), a compound known to be able to shunt electrons to oxygen. Pulse radiolysis data indicate that the reaction of the K_3 radical anion with oxygen is lower than that for the nifuroxime radical anion, although K_3 appears to be more active as an electron-shunting molecule in the cell system. However, this may indicate that metabolic production of the K_3 radical anion, as the rate-limiting step, is faster than that of the nifuroxime radical anion, which correlates well with the observation that the cellular activity of K_3 reductase is much greater than that of nitroreductase.

The demonstration of anaerobic reduction of NF on the microsomes [14], coupled with the fact that isolated microsomes incubated with NF in the presence of glucose and NADPH can account for practically all of the NF-stimulated oxygen uptake in the intact EAT cell suspension (manuscript in preparation), suggests that the stimulation of oxygen consumption by NF is extramitochondrial.

One consequence of nitrofuran radical anions reacting with oxygen will be the production of the superoxide radical anion, O_2^-, which in the presence of superoxide dismutase may react with itself to produce, following protonation, H_2O_2 and O_2. Excess production of superoxide or H_2O_2 would be expected to be damaging to cells and may in part explain the cytotoxic effects of the nitrofurans [5]. Similar interaction with O_2 and the dismutation of the resulting oxygen radical have been demonstrated for the intermediary product in the reduction of K_3 [22], as well as for the proximate carcinogen, 4-hydroxy aminoquinoline *N*-oxide [23].

The reducing equivalents necessary for cellular reduction of NF, which are supplied largely through NAD(P)H, are supplied by either glycolysis or pentose cycle activity. Glutathione may participate as a redox buffer by providing reducing equivalents to NADPH through the glutathione reductase enzyme. This conclusion is further supported by the observation that the increased oxygen consumption in the presence of NF and K_3 is enhanced by the addition of glucose but is diminished or even prevented by the addition of diamide (an agent known to deplete GSH and other endogenous substrates [10]).

Depletion of intracellular GSH may also occur through the shunting of electrons to oxygen by NF with the result that GSSG would accumulate. This would inhibit sulfhydryl enzymes such as succinoxidase involved in mitochondrial oxidations [24]. A direct inhibition of glutathione reductase by NF [7] may also occur. The inhibition of respiration is more pronounced when diamide is added to the cells before NF.

With regard to the use of nitrofuran derivatives as cellular radiation modifiers [1, 2, 5], our results indicate that the compounds are far from metabolically inert and that special attention should be given to the alteration in electron transfer reactions and changes in intracellular reducing species as well as the production of free radicals by the drug, when interpreting results obtained with these agents.

Acknowledgements—We would like to thank Mrs. Birgit Jacobson and Mr. Steve Richie for capable technical assistance.

REFERENCES

1. C. L. Greenstock and I. Dunlop, *Radiat. Res.* **56,** 428 (1973).
2. A. P. Reuvers, J. D. Chapman and J. Borsa, *Nature, Lond.* **237,** 402 (1972).
3. D. R. McCalla, *Can. J. Microbiol.* **11,** 185 (1965).
4. T. Yahagi, N. Miriako, H. Kazuko, T. Matsushima, T. Sugimuia and G. T. Bryan, *Cancer Res.* **34,** 2266 (1974).
5. J. D. Chapman, A. P. Reuvers and J. Borsa, *Br. J. Radiol.* **46,** 623 (1973).
6. J. A. Buzard and K. Kopko, *J. biol. Chem.* **238,** 464 (1963).
7. H. E. Paul and M. F. Paul, in *Experimental Chemotherapy* (Eds. R. J. Schnitzer and F. Fawking), Vol. IV (suppl. to Vol. II), p. 521. Academic Press, New York (1966).
8. E. J. Smith and E. J. Van Loon, *Analyt. Biochem.* **31,** 315 (1969).
9. L. Fishbein, W. G. Flamm and H. L. Falk, *Chemical Mutagens*, p. 44. Academic Press, New York (1970).
10. J. E. Biaglow and O. F. Nygaard, *Biochem. biophys. Res. Commun.* **54,** 874 (1973).
11. J. W. Hunt, C. L. Greenstock and M. J. Bronskill, *Int. J. Radiat. Biol.* **4,** 87 (1972).
12. C. L. Greenstock and I. Dunlop, *J. phys. Chem., Ithaca* **77,** 1834 (1973).
13. J. R. Gillette and T. E. Gram, in *Microsomes and Drug Oxidation* (Ed. J. R. Gillette), p. 133, Academic Press, New York (1969).
14. J. R. Fouts and T. E. Gram, in *Microsomes and Drug Oxidation* (Ed. J. R. Gillette), p. 81. Academic Press, New York (1969).
15. E. E. Gordon, L. Ernster and G. Dallner, *Cancer Res.* **27,** 1372 (1967).
16. C. L. Greenstock and I. Dunlop, *Fedn Proc.* **33,** 1462 (1974).
17. R. L. Willson, *Chem. Commun.* 1005 (1970).
18. E. J. Land and J. Swallow, *Biochim. biophys. Acta* **234,** 34 (1971).
19. I. M. Kolthoff and J. L. Lingane, *Polarography*, 2nd Edn. Vol. II, p. 748. Interscience, New York (1965).
20. J. E. Biaglow, O. F. Nygaard and C. L. Greenstock, *Radiat. Res.* **59,** 158 (1974).
21. J. M. Gillard and G. Tolliu, *Biochem. biophys. Res. Commun.* **58,** 328 (1974).

22. I. Fridovich, in *Molecular Mechanisms of Oxygen Activation* (Ed. O. Hayaiski), p. 468. Academic Press, New York (1974).
23. A. Matsuyama and C. Nagata, in *Topics in Chemical Carcinogenesis* (Ed. W. Nakahara), Proc. Second Int. Symp. of the Princess Takamatsu Cancer Research Fund. 35 (1972).
24. P. C. Jocelyn, *The Biochemistry of the SH Group*, p. 224. Academic Press, New York (1972).