### Title
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### Journal
Neurochemistry International, 17(3)

### ISSN
0197-0186



### Authors
LeBel, Carl P
Bondy, Stephen C



### Publication Date
1990

### DOI
10.1016/0197-0186(90)90025-o

*Neurochem. Int.* Vol. 17, No. 3, pp. 435–440, 1990
Printed in Great Britain. 

0197-0186/90 $3.00 + 0.00


# SENSITIVE AND RAPID QUANTITATION OF OXYGEN REACTIVE SPECIES FORMATION IN RAT SYNAPTOSOMES

CARL P. LEBEL and STEPHEN C. BONDY
Department of Community and Environmental Medicine, Southern Occupational Health Center, University of California, Irvine, CA 92717, U.S.A.

(*Received* 15 *November* 1989; *accepted* 26 *January* 1990)

**Abstract**—The formation of oxygen reactive species in response to oxidative stimuli was measured in rat synaptosomes. Studies employed the non-fluorescent probe 2′,7′-dichlorofluorescin diacetate (DCFH-DA), which after de-esterification is oxidized in the presence of oxygen reactive species to the highly fluorescent 2′,7′-dichlorofluorescein (DCF). Oxygen reactive species formation, as measured by DCF fluorescence, was stimulated by ascorbate and/or $FeSO_4$, and xanthine/xanthine oxidase under various buffering conditions. These agents all increased DCF formation in Tris, HEPES and phosphate buffer. Ascorbate also stimulated the formation of DCF in a concentration-dependent manner. The presence of $Ca^{2+}$ in HEPES buffer did not enhance or diminish the effects of ascorbate/$FeSO_4$ on DCF formation. Deferoxamine inhibited the ascorbate/$FeSO_4$-induced stimulation of DCF formation, but xanthine/xanthine oxidase-induced stimulation was not affected by pretreatment with superoxide dismutase. Results indicate that DCF fluorescence is a sensitive, quantitative and direct measure of oxygen reactive species formation in synaptosomes, providing a rapid method for investigating early neuronal events that occur during oxidative stress.

Numerous studies have documented that formation of free radicals plays a major role in events leading to biological membrane damage (for reviews see Kappus, 1985; Halliwell and Gutteridge, 1984). In the central nervous system, much has been written regarding oxygen radical formation as an early post-traumatic event following spinal cord injury (Demopoulos *et al.*, 1982; Milvy *et al.*, 1973), and in the destruction of catecholamine neurons (Cohen, 1984). Oxidative stress is reported to be magnified by elevations in intracellular $Ca^{2+}$ (Braughler *et al.*, 1985), modulate γ-aminobutyric acid (GABA)/barbiturate receptor function (Schwartz *et al.*, 1988), affect membrane potentials (Lambert and Bondy, 1989), and other electric membrane properties (Scott and Lew, 1988). Using electron spin resonance techniques, brain mitochondrial superoxide radical formation has been shown to be age-related (Sawada and Carlson, 1987).

Traditionally, free-radical initiated lipid peroxidation has been estimated with techniques such as the reaction between thiobarbituric acid and malondialdehyde, and by conjugated dienes. However, thiobarbituric acid and malondialdehyde have been shown to cross react with several endogenous substances (deoxyribose, amino acids) and functional moieties such as amino groups (Gutteridge, 1981; Halliwell and Gutteridge, 1981). Additionally, studies that argue against the use of thiobarbiturate/malondialdehyde-like indicators of free-radical generation report that oxygen radical-induced events such as proteolysis precede, and are independent of, lipid peroxidation (Davies and Goldberg, 1987a, b). Finally, the instability of the thiobarbiturate/malondialdehyde complex in the presence of $H_2O_2$, a highly reactive oxygen species, was recently demonstrated (Kostka and Kwan, 1989).

A direct measurement of oxygen reactive species has been reported in cell culture systems using the non-fluorescent probe DCFH-DA. Relative intracellular oxidation has been quantitated via DCF fluorescence in polymorphonuclear leukocytes (Szejda *et al.*, 1984; Bass *et al.*, 1983) and renal epithelial cells (Scott *et al.*, 1988) in flow cytometric studies. DCFH-DA is a stable, non-fluorescent molecule that readily crosses cell membranes, and is hydrolyzed by intracellular esterases to non-fluorescent 2′,7′-dichlorofluorescin (DCFH) (Bass *et al.*, 1983). DCFH is then rapidly oxidized in the presence of oxygen reactive species to highly fluorescent DCF (Bass *et al.*, 1983; Szejda *et al.*, 1984; Scott *et al.*, 1988).

Since all existing studies investigating the formation of oxygen reactive species have used cell culture systems, our interests were to adapt the use of DCFH-DA to subcellular systems, widely used by biochemists

and neurochemists. Furthermore, DCFH-DA may prove useful as a marker of early neurological insult. A recent study demonstrated that dietary vitamin E deficiency resulted in lower basal formation rates of oxygen reactive species in cerebrocortical P2 fractions using DCFH-DA (LeBel *et al.*, 1989).

In order to expand the potential utility of DCFH-DA, it is necessary to consider some possible limitations: (1) Does DCFH-DA truly reflect intracellular (intrasynaptosomal) oxidative events? (2) Can it be accurately quantitated? (3) What oxidative species are assayed? (4) How sensitive is the analysis? (5) Is DCF fluorescence prone to artifact? This study investigates the application of the DCFH-DA probe to measure oxygen reactive species in rat synaptosomes.

## EXPERIMENTAL PROCEDURES

### *Animals*

Adult male CR 1 CD rats (250–300 g) were obtained from Charles River Breeding Laboratories (Wilmington, Mass.), and were maintained in the animal facility in clear polypropylene cages with water and food provided *ad libitum*.

### *Synaptosomal preparation*

Rats were decapitated, the brains rapidly excised and placed on ice and the whole brain minus the cerebellum and pons-medulla was dissected out. Synaptosomes were prepared by the modification of Dodd *et al.* (1981) using the differential centrifugation method of Gray and Whittaker (1962). Briefly, after homogenization in 10 volumes of ice-cold 0.32 M sucrose, the homogenate was centrifuged (1800 ***g***, 10 min, 0–4°C) and the supernatant layered over 1.2 M sucrose (10 ml). After high speed centrifugation (252,000 ***g***, 25 min, Beckman model L8-70, rotor Ti60) the layer at the interface was collected, diluted 2.5-fold with 0.32 M sucrose and layered over 0.8 M sucrose. After centrifugation (252,000 ***g***, 25 min), the synaptosomal pellet was suspended in HEPES buffer, pH 7.4, to give a tissue concentration of 0.15 g-equiv./ml (approx. 1.6 mg/ml protein). The composition of HEPES buffer was (mM): NaCl, 120; KCl, 2.5; $NaH_2PO_4$, 1.2; $MgCl_2$, 0.1; $NaHCO_3$, 5.0; glucose, 6.0; $CaCl_2$, 1.0 and HEPES 10.

### *Protein determination*

Protein content of synaptosomes was assayed by the method of Bradford (1976) using bovine serum albumin as a reference.

### *Assay for oxygen reactive species formation*

Synaptosomes (0.5 ml) were diluted in 9 vols of either HEPES, HEPES minus $CaCl_2$, 40 mM Tris or 0.1 M $NaH_2PO_4$, all at pH 7.4. The diluted synaptosomes (5.0 ml) were then incubated with 5 $\mu$M DCFH-DA (added from a stock solution of 1.25 mM in methanol) at 37°C for 15 min. To terminate the incubation, the synaptosomes were centrifuged at 12,500 ***g*** for 8 min (0–4°C), and the pellet was resuspended in 5 ml of the respective ice-cold buffer. Fluorescence was monitored on a Farrand Spectrofluorometer, with excitation wavelength at 488 nm (band width 5 nm), and emission wavelength 525 nm (band width 20 nm). The cuvette holder was thermostatically maintained at 37°C.

Autofluorescence of synaptosomes was corrected for by the inclusion in each experiment of parallel blanks (unloaded synaptosomes). The correction for autofluorescence was always less than 11% of the total. Oxygen reactive species formation was quantitated from a DCF standard curve in methanol (0.05–1.0 $\mu$M).

After loading with DCFH-DA, the synaptosomes were incubated in either ascorbate (0.01–1.0 mM), $FeSO_4$ (5 $\mu$M), ascorbate (0.1 mM)/$FeSO_4$ (5 $\mu$M), or xanthine (0.25 mM)/xanthine oxidase (0.075 U/ml). For inhibition studies, deferoxamine (0.1 mM) was added simultaneously with ascorbate/$FeSO_4$, while superoxide dismutase (300 U/ml) was added 5 min prior to addition of xanthine/xanthine oxidase. The concentrations of oxidative stimuli used in this study are comparable to reports investigating oxidative damage (Braughler *et al.*, 1985; Davies and Goldberg, 1987a). Fluorescence measurements were taken immediately (prior to stimulant addition) and 1, 5, 15, 30 and 60 min after stimulation.

DCFH was prepared from DCFH-DA by the method of Cathcart *et al.* (1984) by mixing 0.5 ml 1.0 mM DCFH-DA in methanol, with 2.0 ml 0.01 N NaOH. This de-esterification of DCFH-DA proceeded at room temperature for 30 min, then was neutralized with 10 ml 25 mM $NaH_2PO_4$, pH 7.4. This solution was kept on ice in a foil wrapped container until analysis. To determine whether the incubation media alone oxidized DCFH, 50 $\mu$l of DCFH was placed in 1.95 ml Tris, the fluorescence was recorded, the solution was incubated at 37°C for 60 min and the final fluorescence was determined.

### *Materials*

2′,7′-Dichlorofluorescin diacetate (DCFH-DA) was purchased from Molecular Probes Inc. (Eugene, Or.), while 2′,7′-dichlorofluorescein (DCF) was obtained from Polysciences Inc. (Warrington, Pa). $FeSO_4$ was purchased from Fisher Scientific (Fair Lawn, N.J.). All other chemicals were obtained from Sigma Chemical Co. (St Louis, Mo.).

## RESULTS

### *(a) Dependence of probe fluorescence on biological tissue*

DCFH-DA was placed in Tris buffer in the absence of synaptosomes to determine whether intrasynaptosomal esterases were required for the eventual oxidation of DCFH-DA to DCF. No fluorescence was observed either prior to or following the addition of ascorbate/$FeSO_4$ in this aqueous medium. These studies were based on the assumption that DCFH oxidation is solely due to biological processes and does not occur significantly in the presence of the incubation medium. In order to verify this assumption, DCFH was prepared from DCFH-DA (see Experimental Procedures section) and its proneness to oxidative media alone was studied. The rate of intrinsic oxidation of DCFH was invariably below 3%

of the corresponding rate in the presence of biological tissue (Table 1). This was the case even in the presence of potentially oxidizing environments containing 0.1 mM ascorbate/5 $\mu$M $FeSO_4$.

To determine the degree of leakage by DCFH from synaptosomes, DCFH-DA loaded samples were incubated for 60 min and the fluorescence recorded. The samples were recentrifuged for 8 min at 12,500 ***g***, resuspended in the same volume of Tris, and the fluorescence was recorded. Synaptosomes contained from 75 to 82% of the original DCF formed, or an approximate 18–25% leakage of DCFH formed intrasynaptosomally.

*(b) Synaptosomal production of oxygen radicals*

Unstimulated synaptosomes pre-loaded for 15 min with DCFH-DA in several buffers showed detectable basal levels of DCF fluorescence after a 60 min incubation (Table 2). Basal levels of DCF formation in phosphate and Tris buffers were somewhat higher than in both HEPES buffers. In all of the buffers employed, ascorbate was more efficacious in stimulating DCF formation than was $FeSO_4$, while $FeSO_4$ alone was unable to induce DCF formation in phosphate and HEPES–$Ca^{2+}$ buffers.

In all buffers, the combination of ascorbate and $FeSO_4$ potentiated the formation of DCF, as compared to incubations with ascorbate and $FeSO_4$ alone (Table 2). Ascorbate/$FeSO_4$-stimulated DCF formation was higher in phosphate and Tris buffers than in $Ca^{2+}$-containing or $Ca^{2+}$-absent HEPES buffers. The presence of $Ca^{2+}$ in HEPES did not enhance or diminish the effects of ascorbate/$FeSO_4$ on DCF formation.

Synaptosomes were pre-loaded with DCFH-DA for various times prior to ascorbate/$FeSO_4$ stimulation. Maximal DCF formation was reached after pre-loading for 15 min (Fig. 1).

The rate of DCF formation was linear ($r = 0.986$) with respect to the amount of protein employed (Fig. 2). Ascorbate/$FeSO_4$-induced DCF formation could be detected using as little as 40 $\mu$g synaptosomal protein.

The generation of DCF in synaptosomes, when stimulated by ascorbate, was also concentration-dependent (Fig. 3). This ascorbate/$FeSO_4$-stimulated DCF formation was completely blocked by deferoxamine (Fig. 4). While xanthine/xanthine oxidase

Table 1. The oxidation of non-fluorescent DCFH to fluorescent DCF in the presence and absence of synaptosomes

| | DCF formation (pmol/2 ml/min) | |
|---|---|---|
| Media | −Synaptosomes* | +Synaptosomes† |
| Tris alone | 2.1 | 69.3 |
| Tris + ascorbate/$FeSO_4$ | 7.1 | 262.1 |

Incubations were in the presence of either exogenously added DCFH* or synaptosomally generated DCFH† at a concentration of 0.7–1.0 $\mu$M, and 0.16 mg/ml synaptosomal protein was employed. The data are expressed as the mean DCF formed in pmol/2 ml/min cuvette and were obtained from two independent experiments with differences no larger than 7% between assays.

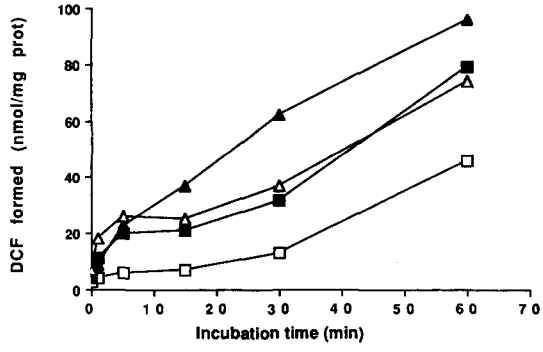


Fig. 1. Ascorbate/$FeSO_4$-induced DCF formation after various dye loading times. Data were obtained from two independent experiments and are expressed as the mean with differences no larger than 9% between assays. □, 5 min; ▲, 15 min; ■, 30 min; △, 60 min.

Table 2. The effect of several incubation buffers on synaptosomal DCF formation in the presence of several oxidizing conditions

| | DCF formation (pmol/mg prot/min) | | | |
|---|---|---|---|---|
| | Buffer used | | | |
| Treatment | Phosphate | Tris | HEPES + $Ca^{2+}$ | HEPES − $Ca^{2+}$ |
| Basal | 78 | 67 | 50 | 37 |
| Ascorbate | 1878 | 743 | 253 | 649 |
| $FeSO_4$ | 88 | 470 | 161 | 17 |
| Ascorbate/$FeSO_4$ | 2445 | 2218 | 1445 | 1338 |

Synaptosomes were exposed to various oxidizing agents in several buffers at 37°C for 60 min. The data were obtained from two independent experiments and are expressed as the means with differences no larger than 11% between assays.

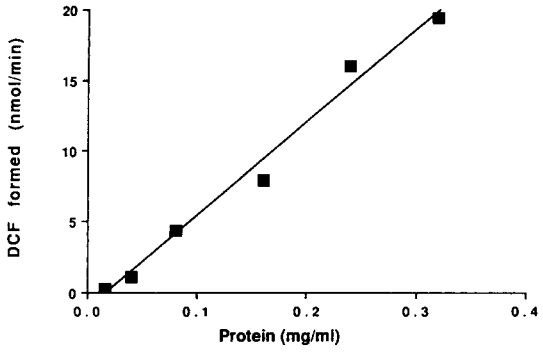


Fig. 2. The relation of protein concentration to ascorbate/$FeSO_4$-induced DCF formation. The incubation time was 60 min. Data were obtained from two independent experiments and are expressed as the mean with differences no larger than 11% between assays.

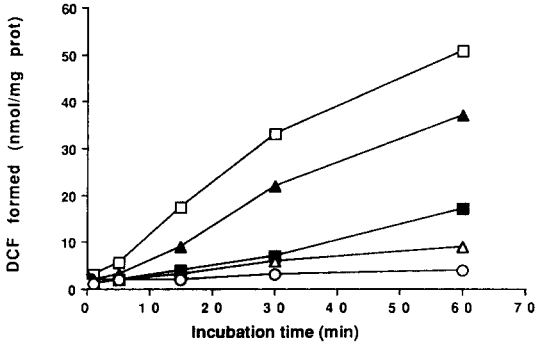


Fig. 3. The concentration-response effects of ascorbate on DCF formation. Data were obtained from two independent experiments and are expressed as the mean with differences no larger than 9% between assays. □, 100 μM ascorbate; ▲, 10 μM ascorbate; ■, 1 μM ascorbate; △, 0.1 μM ascorbate; ○, basal.

induced excess DCF formation, this stimulation was not inhibited by superoxide dismutase.

## DISCUSSION

Biochemical reactions and pathways that utilize oxygen produce reduced oxygen species such as superoxide anion ($O_2^-$), hydrogen peroxide ($H_2O_2$), and hydroxyl radical (·OH) (Kappus, 1985; Halliwell and Gutteridge, 1984). These oxygen reactive species have recently been the subject of much attention in the CNS (Cohen, 1984; Braughler *et al.*, 1985; Schwartz *et al.*, 1988; Scott and Lew, 1988). To date however, the oxidation of spin trapping agents by $O_2^-$ (measured

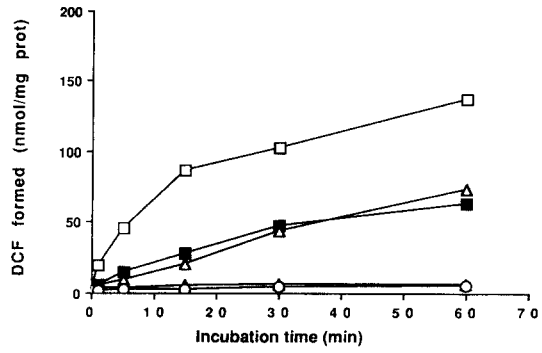


Fig. 4. The effects of oxygen radical generating agents and inhibitors on DCF formation. Data were obtained from two independent experiments and are expressed as the mean with differences no larger than 10% between assays. □, Ascorbate/$FeSO_4$; ▲, ascorbate/$FeSO_4$+deferoxamine; ■, xanthine/xanthine oxidase; △, xanthine/xanthine oxidase +superoxide dismutase; ○, basal.

by ESR spectroscopy) is the only direct measure for basal levels of oxygen reactive species employed in neuronal tissues (Sawada and Carlson, 1987).

The intracellular trapping of DCFH, and its subsequent oxidation to DCF has been reported as a direct measure of oxygen reactive species formation in non-neuronal tissue (Scott *et al.*, 1988; Szejda *et al.*, 1984; Bass *et al.*, 1983). The techniques employed in the present study demonstrate that the non-fluorescent chemical (DCFH) is largely trapped within the synaptosome as a result of intrasynaptosomal de-esterification. As noted in the methods, following preloading with DCFH-DA, synaptosomes were centrifuged (pelletted) and resuspended in fresh buffer, which allowed for the removal of extrasynaptosomal DCFH-DA. The presence of synaptosomes was required for de-esterification of DCFH-DA to the activated substrate DCFH since no fluorochrome was detected in Tris buffer alone. Virtually all the oxygen reactive species described here were genuinely formed within biological tissue, since without such tissue, DCF formation occurred at a very low rate (Table 1). DCFH leakage (18–25%), is similar to that observed for the $[Ca^{2+}]_i$ indicator dye fura-2 AM in synaptosomes (Bondy and McKee, 1990). These data support the concept that DCFH-DA enters synaptosomes, is de-esterified by intrasynaptosomal esterases to DCFH, which is oxidized by intrasynaptosomally formed oxygen reactive species to the detected fluorochrome DCF.

Under basal conditions, DCF formation was relatively low (Table 2) while after addition of free radical

generating agents, DCF formation increased (Figs 1, 3 and 4). The fact that DCF formation showed good linearity with the amount of protein employed (Fig. 2), and was stimulated by ascorbate in a concentration-dependent manner (Fig. 3) further supports the use of DCFH-DA as an oxygen reactive species probe in neuronal tissue preparations.

The optimal dye loading time that provided maximal DCF formation after ascorbate/$FeSO_4$ stimulation was 15 min (Fig. 1). Interestingly, the extent of DCF formation was: 15 min > 60 min ⩾ 30 min > 5 min. Since DCF fluorescence reflects intracellular oxygen reactive species formation, lengthy dye loading times (30–60 min) may allow for increased dye leakage from the synaptosome. Therefore, beyond 15 min, DCFH may increasingly leak into the extrasynaptosomal medium and is removed consequently by centrifugation, resulting in lower rates of synaptosomal DCF formation.

Ascorbate/$FeSO_4$-induced DCF formation was completely inhibited by deferoxamine (Fig. 4), a specific $Fe^{3+}$ chelator. These findings demonstrate that DCF (oxygen reactive species) formation results from iron-dependent radical reactions (Halliwell and Gutteridge, 1986). Ascorbate/$FeSO_4$ and xanthine/xanthine oxidase are known to produce $O_2^{\cdot-}$, $H_2O_2$ and ·OH (Davies and Goldberg, 1987b; Kuppusamy and Zweier, 1989). Xanthine/xanthine oxidase-induced DCF formation was not blocked by superoxide dismutase (Fig. 4), a result in agreement with a report of Bass and coworkers (1983) who were unable to inhibit xanthine/xanthine oxidase-induced DCF formation in neutrophils. Thus, $O_2^{\cdot-}$ does not appear to play a role in the oxidation of DCFH to DCF in oxidatively stressed synaptosomes. Alternatively, exogenously added superoxide dismutase may have been unable to penetrate the intact synaptosomal membrane, preventing its interaction with $O_2^{\cdot-}$, as suggested by Chan *et al.* (1988).

Our results suggest that in oxidative stress studies, care must be taken with regard to the choice of buffer. While Tris and phosphate buffers enabled maximal DCF formation after ascorbate/$FeSO_4$ stimulation, $FeSO_4$ alone did not stimulate DCF formation in phosphate buffer, perhaps due to formation of an insoluble iron–phosphate complex (Table 2). HEPES buffer attenuated ascorbate/$FeSO_4$-stimulated formation of DCF. Recent studies have reported that Tris and Hepes buffers may afford protection against oxygen radical-induced proteolysis of bovine serum albumin (Davies *et al.*, 1987). This study partially confirms the concept that HEPES can quench the reactivity of free radicals. In the present study, Tris provided little protection against oxygen reactive species (Table 2). Although Tris has been suggested to be a good scavenger of ·OH, substitution of Tris with phosphate buffer did not enhance the formation of DCF (Table 2). Furthermore, studies from this laboratory found that well known ·OH scavengers, such as DMSO (1%, v/v) and mannitol (0.1 mM) decreased DCF formation in Tris buffer (data not shown). These findings support the hypothesis that DCFH is oxidized by more than one form of oxygen reactive species, such as $H_2O_2$, ·OH and ferryl ion (Bass *et al.*, 1983; Szejda *et al.*, 1984; Dunford, 1982).

Much has been written regarding the role of $Ca^{2+}$ in mediating cytotoxic mechanisms in the brain (Patel *et al.*, 1988; Komulainen and Bondy, 1988). To determine whether $Ca^{2+}$ plays a role in DCF formation, ascorbate/$FeSO_4$-induced DCF formation in HEPES buffer with and without $Ca^{2+}$ was investigated. $Ca^{2+}$ did not significantly enhance the effects of ascorbate/$FeSO_4$ on DCF formation (Table 2). Therefore, $Ca^{2+}$ does not appear to play a role in the intracellular formation of oxygen reactive species, as measured by DCF formation, in synaptosomes. Other studies report that $Ca^{2+}$ and free radicals act synergistically in lipid peroxidation to damage cell membranes (Braughler *et al.*, 1985). Braughler and coworkers measured the effects of free radicals on synaptosomal GABA uptake, an indirect measure of lipid peroxidation. The present study makes no statement regarding lipid peroxidation, but directly measures oxygen reactive species generation (DCF formation), which is generally believed to be the initiation step in lipid peroxidation. Therefore, this lack of a synergistic effect by $Ca^{2+}$ in ascorbate/$FeSO_4$-induced DCF formation supports the hypothesis that early oxygen radical-induced damage may be independent of lipid peroxidation (Davies and Goldberg, 1987a; Richards *et al.*, 1988). On the other hand, our data show no exacerbation of oxidative activity in the absence of $Ca^{2+}$ in the incubation medium. Such a $Ca^{2+}$-free milieu has been postulated to enhance free radical formation and thus bring about cell death (Fariss and Reed, 1985).

This study constitutes the first report concerning the application of a fluorescent technique for quantitation of oxygen reactive species formation in synaptosomes. The method is rapid, quantitative, and since DCF formation is in direct molar proportion to oxygen reactive species (i.e. ·OH, $H_2O_2$, ferryl ion), the results may be expressed directly as moles of oxygen reactive species formed. The sensitivity of the fluorescent technique allows quantitation of basal formation rates of oxygen reactive species, and thus has

the potential to be adapted for studies involving *in vivo* exposure to neurotoxic compounds (LeBel *et al.*, 1989). The sensitivity also allows for further adaptation of the method to subcellular preparations in separate brain regions. The use of DCF should provide further insight into the early events that occur under oxidative stress conditions in the brain.

*Acknowledgement*—This work was supported by NIH Grants No. ES04071 and No. ES07157.

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