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# EPR method for the measurement of cellular sulfhydryl groups

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An EPR method that can measure the concentration of sulfhydryl groups in intact cells has been developed using a specially designed stable nitroxyl biradical. The biradical, *ṘS–SṘ*, contains a disulfide bond and readily undergoes thiol–disulfide exchange reactions with thiols resulting in a characteristic EPR spectrum which can be analyzed to provide a quantitative measure of sulfhydryl groups. The data obtained from the EPR method are in good agreement with those obtained from the conventional optical method using Ellman's reagent. The advantages of the EPR method are that the measurement can be carried out on intact cells or any other highly colored, absorbing and/or scattering solutions and the sensitivity is such that only a few cells (~ 100) are needed for each quantitative measurement.

Nitroxyl biradical; Thiol–disulfide exchange; EPR; Intact cell; Glutathione content

## 1. INTRODUCTION

Glutathione (GSH), a natural tripeptide, occurs in all mammalian tissues at high concentration (1–5 mM) (see e.g. [1]), primarily in its reduced form. It is the main SH-containing, low molecular weight component of living tissues. Of the numerous biological functions of GSH [2,3], the two most important functions are: removing toxic metabolites, including peroxides, from the cell and maintaining cellular sulfhydryl groups in their reduced form. Many abnormalities and diseases are due to changes in GSH levels [2,3]. In this connection the quantitative determination of GSH in living tissues is one of the most important methodical and scientific problems. Optical and chromatographic methods have been used to determine the SH groups quantitatively [4] but there are disadvantages associated with these methods. The optical methods require that the sample be optically transparent so they are difficult to apply with highly scattering, absorbing or colored systems such as cell suspensions. Chromatographic methods are rather labor-consuming and are not well-suited to rapid analysis. The proposed NMR method is not sensitive enough for many uses [5].

Recently we have proposed a new method for the quantitative measurement of SH groups in low- and high-molecular weight compounds based on EPR spectroscopy [6]. The method is based on thiol-disulfide exchange reactions of a stable nitroxide biradical containing a disulfide bond, *ṘS–SṘ* (Fig. 1), with SH groups of thiols. This method has been used to determine concentrations of cysteine and GSH in the blood of mice and rats [6] and for measuring acetylcholinesterase activity in homogenate obtained from insects [7].

In this paper we report on the extension of the EPR method to cells, measuring GSH in intact Chinese hamster ovary (CHO) cells. The proposed method is shown to be consistent, within experimental error, with the method using the Ellman reagent [8]. Quantitative measurement of GSH could be made with only 100 CHO cells.

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## 2. MATERIALS AND METHODS

*ṘS–SṘ* was synthesized as described in [6]. Since *ṘS–SṘ* is hydrophobic, it was dissolved in DMSO and added to the sample with the final concentration of DMSO less than 5%.

The CHO cell line was a gift from Dr L. Hopwood, Medical College of Wisconsin and has been maintained in our laboratory for several years. The cells were grown to confluence at 37°C in McCoy's 5a medium and collected by trypsination. The cells ($2 \times 10^5$/ml) were then incubated at 37°C for 24 h in a Bellco spinner flask just prior to their use in these experiments.

For the routine measurement of SH groups in intact cells, $2.5 \times 10^6$ cells were suspended in 1.5 ml phosphate buffer solution containing $8 \times 10^{-5}$ M *ṘS–SṘ* and the EPR spectrum was taken after 3 min. For measurements on frozen–thawed cells, $2.5 \times 10^6$ cells were frozen–thawed 3 times, then $8 \times 10^{-5}$ M *ṘS–SṘ* was added and the EPR spectrum was taken after 3 min. For the measurement of SH groups in the aqueous compartment of cells, frozen–thawed cell suspensions were centrifuged at $6000 \times g$ then $8 \times 10^{-5}$ M *ṘS–SṘ* was added to the supernatant. In each EPR measurement, a small portion (100 $\mu$l) of each preparation was placed into a gas-permeable Teflon tube (Zeus Industries, Raritan, NJ), 1 mm in diameter, 10 cm long, folded in half and placed in a quartz tube open on both ends, the quartz tube was placed in the horizontal EPR cavity.

For measurement of the total SH groups in CHO cells, $2.5 \times 10^6$ cells were freeze-thawed 3 times and 0.5 mM Ellman's reagent was added to reach a total volume 1.5 ml. The supernatant was taken after

centrifugation at 6000 × g and the optical density was measured at 412 nm. For measurement of SH groups in the aqueous cellular compartment, Ellman's reagent was added to the supernatant after the centrifugation and the optical density of 412 nm was measured ($\varepsilon_{412}$ = 1.4 × $10^4$ $M^{-1}\cdot cm^{-1}$ [9]).

Cellular SH groups also were measured by both EPR and optical methods after incubation of CHO cells with L-buthionine-S,R-sulfoximine (BSO) (purchased from Chemical Dynamics), which inhibits glutatione synthesis [10]. 2.5 ×$10^6$ cells in 1 ml medium were incubated with 1 mM BSO at 37°C for 7 h and washed 3 times with phosphate buffer solution; then the cellular SH groups were measured by the methods described above.

All EPR spectra were taken at room temperature (22°C) on a Varian E-109 spectrometer equipped with an EPR data acquisition system [11]. The usual instrumental parameters were: microwave frequency, 9.05 GHz; incident microwave power, 5 mW; center of the field, 3210 G; scan range, 100 G; and field modulation, 1 G. Optical measurements were taken at room temperature (22°C) on a Hewlett Packard 8452A Diode Array Spectrophotometer equipped with data acquisition system.

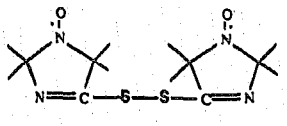
The test of sensitivity of the EPR method was done in a flat quartz cell to achieve maximal sensitivity. Concentration of both cells and *ṘS–SṘ* were gradually decreased to the levels where the EPR signal and its change due to reactions between cellular SH groups and the biradicals could be observed at a signal-to-noise ratio around 4:1. A receiver gain of $10^5$, modulation amplitude of 4 G and incident microwave power of 50 mW were used to optimize the signal.

## 3. RESULTS AND DISCUSSION

The biradical *ṘS–SṘ* participates in thiol–disulfide exchange reactions with thiols resulting in the change of its EPR spectrum because of the production of the monoradicals, *Ṙ–SH* and *BS–SṘ* [6,7]:

$$\dot{R}S\text{–}S\dot{R} + B\text{–}SH \underset{K_r}{\overset{K_f}{\rightleftharpoons}} R\text{–}SH + BS\text{–}SR \qquad (1)$$

The difference in the EPR spectra of *ṘS–SṘ*, *R–SH* and *BS–SṘ* forms the basis for the EPR method (Fig. 2). For [*ṘS–SṘ*] > [*B–SH*] the equilibrium in reaction (1) is shifted to the right-hand side and an increase in EPR signal intensity of the monoradicals formed can be used for the quantitative determination of GSH in the sample. As has been shown in [6] an increase in signal intensity of a component of the spectrum due to the monoradical is approximately 16 times greater than a decrease in the signal intensity of component of the EPR spectrum due to biradical. The double-integral of the spectrum remains unchanged [6]. Therefore, measurement of the relative increase of the peak height of the monoradical component upon addition of SH containing low molecular weight compounds to the system is a very sensitive method for the determination of their absolute concentration.



ṘS-SṘ

Fig. 1. Chemical structure of the biradical *ṘS–SṘ*.

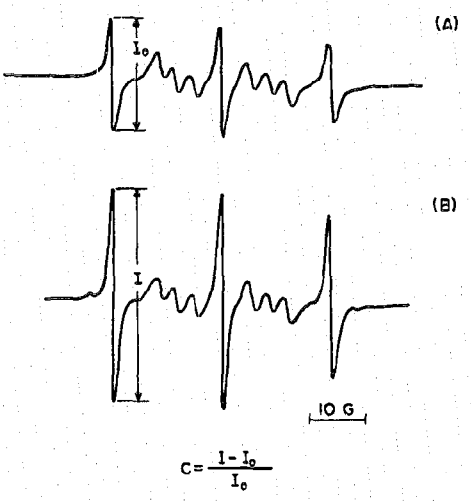



Fig. 2. Change of the EPR spectrum of *ṘS–SṘ* after its reaction with SH. Spectrum A was from 8 × $10^{-5}$ M *ṘS–SṘ* alone in phosphate buffer solution (pH 7.3) and spectrum B was from the same solution after 1.6 × $10^{-5}$ M GSH was added. The EPR instrumental parameters were as described in section 2.

Fig. 3 shows the relationship between the EPR parameter *C*, defined in Fig. 2, and the concentration of sulfhydryl groups of glutathione. We have found experimentally that the linear dependence of the parameter *C* on concentration of GSH is retained up to the ratio [*ṘS–SṘ*]/[*GSH*] ≈ 3. As this ratio decreases (the concentration of GSH increases) the dependence of $(I - I_0)/I_0$ deviates from linearity, which reflects the influence of the back reaction ($K_r$) in thiol–disulfide exchange (see Eqn. 1). As is clearly seen in Fig. 3, at a fixed concentration [*ṘS–SṘ*[ (in our case 8 × $10^{-5}$ M) GSH can be reliably measured within the range 1–25 μM. In addition, no reduction of *ṘS–SṘ* by GSH was found.

Table I shows the results of the comparative study in which the SH groups associated with CHO cells were measured by both EPR and optical methods after different treatments of the cells. The results from the two methods are in good agreement. It is obvious that one of the advantages of the EPR method is that it could be applied to an intact cell suspension or any other highly colored, absorbing or scattering system. The data from the intact cells by EPR are not statistically different

Table I

Measurement of cellular SH groups by EPR and optical methods
Unit: $10^9$ SH groups/cell ± SD (number of experiments)

| Treatment | EPR method | Optical method |
|---|---|---|
| Intact cells | 4.8 ± 1.4 (4) | ---------- |
| Freeze-thawed cells | 4.0 ± 0.7 (4) | 4.7 ± 0.3 (2) |
| Supernatant after freeze-thawing | 3.2 ± 0.2 (4) | 3.6 ± 0.4 (2) |

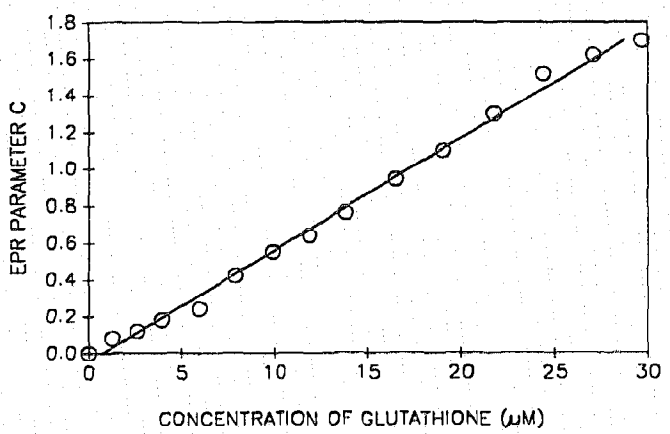



Fig. 3. Effect of GSH on the empirical EPR parameter C of biradical *ṘS–SṘ*. GSH was added gradually to the phosphate buffer (pH 7.3) which contained $8 \times 10^{-5}$ *ṘS–SṘ* and then an EPR spectrum was taken at each given concentration of GSH. The EPR instrumental parameters were as described in section 2.

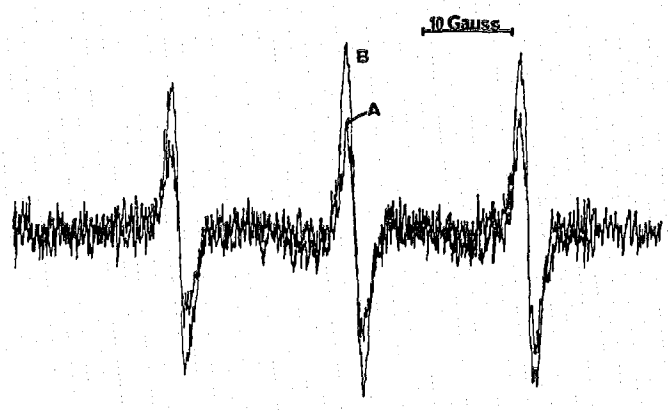



Fig. 4. Test of sensitivity of the EPR method. The spectra were taken from a flat cell which contained $0.25 \times 10^{-6}$ M *RS–SR* in phosphate buffer (pH 7.3). Spectrum A was taken without cells and spectrum B was taken with 130 CHO cells in the flat cell. The setting receiver gain $10^5$; modulation 4 Gauss; incident microwaver power 50 mW; these settings were selected to increase the signal-to-noise ratio. The other EPR parameters were as described in section 2.

from data from the frozen-thawed cells, by both EPR and optical methods, which indicates that the biradical *ṘS–SṘ* readily penetrated into the cells: this was expected because it is uncharged. The data by both EPR and optical methods from the supernatants after freezing-thawing the cells indicate that the SH groups associated with cell membranes and membrane proteins contributed 20–30% to the total cellular SH groups measured on both the intact and frozen-thawed cells.

To determine whether a decrease in cellular GSH level would give similar results with the two methods, we employed BSO, which inhibits GSH biosynthesis and reduces the level of GSH in cells (Table II). Again the results from the two methods were in good agreement; the total cellular SH groups were reduced by 20–30% and the results were reproducible. The cells were counted both before and after incubations with BSO and no changes in cell numbers were found.

To estimate the abolute sensitivity of the method we used a minimal concentration of *ṘS–SṘ* and recorded EPR spectra using a flat cell configuration (Fig. 4). Spectrum A is from 0.25 μM *ṘS–S–R* alone in buffer and spectrum B is taken after about 130 cells are added. Despite the signal-to-noise ratio of about 4:1 in spectrum A, on addition of the cells an increase in peak height of the monoradical component is reproduced reliably. Using the calibration curve, we have estimated the content of GSH in the sample to be about 0.027 μM, so the estimated cellular concentration of GSH is about 5 mM.

A potential limitation of the use of nitroxide for quantitative measurements in cells and tissues is their possible reduction to diamagnetic hydoxylamines [12,13]. We found, however, that under our usual conditions ($2.5 \times 10^6$ CHO cells in 1.5 ml medium and $8 \times 10^{-5}$ M *ṘS–SṘ*), only about 4% of *ṘS–SṘ* was reduced after 5 min, which would not affect the measurement of cellular SH groups by the EPR method.

In summary, the EPR method developed here for the measurement of cellular SH groups produced data consistent with those obtained by optical method. The advantages of the EPR methods are that the measurements can be carried out on intact cells or any other highly colored, absorbing and scattering solutions and only a few cells (~ 100) are needed in each measurement. This method opens up the possibility of determining GSH concentrations in individual cell compartments, the occurrence of such changes have been suggested in some pathological conditions such as Parkinson's disease [14].

Table II

Measurement of cellular SH groups after incubation with BSO
Unit: $10^9$ SH groups/cell ± SD (number of experiments)

| Treatment* | EPR method | Optical method |
|---|---|---|
| Control | 4.2 ± SD 0.3 (2) | 3.5 ± SD 0.1 (2) |
| Incubated with 1 mM BSO for 7 h | 2.6 ± SD 0.1 (2) | 2.9 ± SD 0.0 (2) |

*Cells were freeze-thawed three times prior to the measurement.

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