

THE ESTIMATION OF RED CELL SUPEROXIDE DISMUTASE ACTIVITY BY PULSE RADIOLYSIS IN NORMAL AND TRISOMIC 21 SUBJECTS

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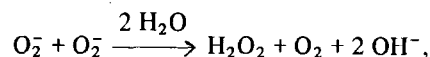
1. Introduction

In Down's syndrome, resulting from an abnormality of chromosome number, trisomy 21 [1], it is assumed that the extra-chromosome is the direct cause of the described morphological and biochemical changes. Numerous reports have appeared describing various enzymatic changes in such patients, but the mechanism by which they are produced as well as the localisation of the structural gene on chromosome 21 are unknown. However a direct relationship between chromosome 21 number and the amount of enzyme is the simplest hypothesis.

Since the gene for cytosol superoxide dismutase (SOD₁) has been assigned to chromosome 21 using mouse-human somatic cell hybrids [2], it was interesting to study this enzyme in Down's syndrome. The use of indirect assay systems [3,4] as well as immunological estimations [5], strongly suggested a proportional increase in the amount of synthesized enzyme. So we proposed to develop a direct assay method of erythrocyte superoxide dismutase using pulse radiolysis.

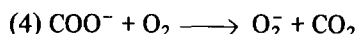
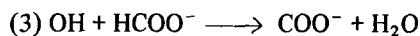
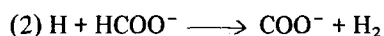
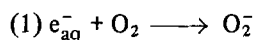
Cytosol superoxide dismutase has been isolated

from a wide range of cells including human erythrocytes and has been finally identified as erythrocuprein, the erythrocyte copper-containing protein [6,7]. This enzyme catalyses the reaction:

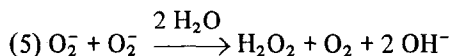


and it has been assumed to be an essential defence against the potential toxicity of oxygen [8].

In our method, high initial concentrations of O_2^- ($\sim 10^{-4}$ M) may be generated by irradiating oxygenated aqueous formate solutions at pH 9. It is known [9–11] that, under these conditions, all primary radiolytic species are transformed, within less than 1 μs , into O_2^- according to the following reactions:



The yield of O_2^- for 100 absorbed eV is $G(O_2^-) = 5.8$. At pH 9, O_2^- disappears slowly by the reaction:



$$(k < 10^2 M^{-1} \cdot s^{-1}).$$

The addition of SOD catalyses this reaction and the disappearance of O_2^- becomes faster [12–14]. O_2^- presents a characteristic band in the ultraviolet region with $\lambda_{max} = 245 \text{ nm}$ ($\epsilon_{245} = 2000 M^{-1} \cdot \text{cm}^{-1}$); its decay can be measured at this wavelength.

2. Experimental

Blood from 9 normal and 8 trisomic 21 children was collected in heparin. Plasma and white cells were removed by centrifugation. The red cells were washed three times with 0.9% NaCl and hemolyzed by adding one volume of bidistilled water. The hemoglobin concentration was measured by the method of Drabkin and adjusted to 10 g per 100 ml with bidistilled water. Hemoglobin was then removed by chloroform–ethanol [15] as follows: 0.5 ml of cold ethanol and 0.3 ml of cold chloroform were added to 2 ml of hemolysate. The mixture was shaken for 30 min and then diluted with 0.3 ml of cold water. The precipitate was removed by centrifugation. 1 ml of the clear supernatant was freeze-dried and then diluted with 20 ml of $10^{-3} M$ sodium borate buffer (pH 9) containing $10^{-1} M$ sodium formate.

The source of pulsed electrons was a modified Febetron 707 used at CEN Saclay (DRA-SRIRMa). In this apparatus the discharge from a series of capacitor-inductance modules is applied to the field emission cathode of a vacuum tube. A magnetic selection of 1.8 MeV electrons is then used to reduce the total pulse length to about 20 ns [16]. The solutions contained in a spectrosil cell ($2.5 \times 2.5 \times 0.25 \text{ cm}$) were irradiated uniformly. At each pulse, the energy absorbed by the solution was about 10^{18} eV/ml , so that the initial concentration of O_2^- produced was about $10^{-4} M$.

The spectrophotometric analysis was carried out with a standard device including a xenon lamp, a monochromator and a photomultiplier. The analysing light-path in the solution was 2.5 cm. The changes of the absorption of O_2^- ($\lambda = 245 \text{ nm}$, $\epsilon = 5000 M^{-1}$)

with time were recorded photographically using an oscilloscope (Tektronix 454), the start of the pulse being taken as the origin of the time scale. All measurements were made at room temperature ($\sim 23^\circ\text{C}$).

3. Results

Figure 1 shows typical oscilloscope traces for a solution with SOD (curve A), for a sample of a normal control (curve B) and a trisomic 21 child (curve C). In the absence of SOD, the variation in O_2^- concentration is very small and can be neglected, thus avoiding the need for a correction. For all measurements, $\log(\text{O.D.})$ is a linear function of time (fig. 2). Thus the O_2^- decay, in the presence of enzyme, corresponds to a first order disappearance kinetics, $\log(\text{O.D.}) = -k [\text{SOD}_1] t + C^{\text{te}}$.

The slopes $k [\text{SOD}_1]$ obtained from the various experiments are given in table 1. The Henry's diagrams (fig. 3) indicate that the distributions of $k [\text{SOD}_1]$ in these two populations are gaussian and distinguishable. In this set of experiments the $k [\text{SOD}_1]$ mean values for normal and trisomic 21 subjects are 58.6 and 82.8 respectively. The values of standard deviations σ , 4.6 and 7.8, lead to 95% confidence intervals ($t \frac{\sigma}{\sqrt{n}}$) of 3.5 and 6.5. The results are:

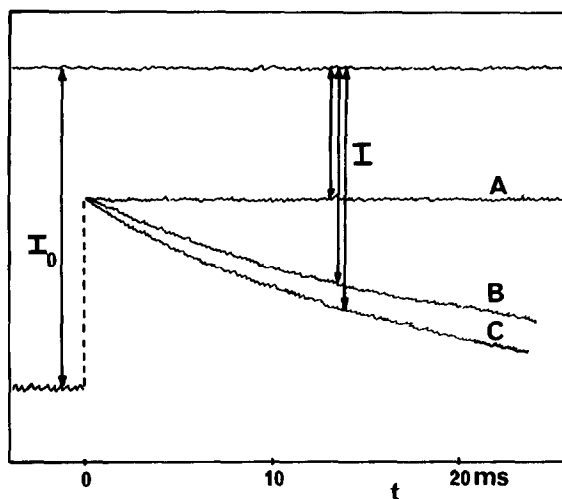


Fig. 1. Oscilloscope traces for: (A) a solution without SOD; (B) sample from a normal child, and (C) sample from a trisomic 21 child.

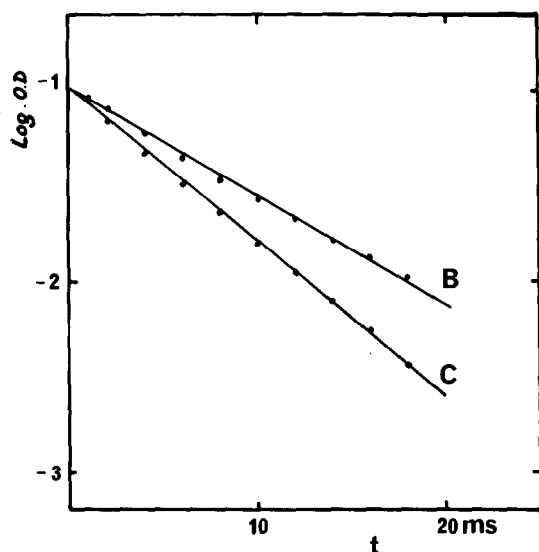


Fig. 2. Semi logarithmic plot of decay of O.D. at 245 nm in the presence of red cell extract from a normal subject (B) and from a trisomic 21 subject (C).

$$(k [\text{SOD}_1])_{\text{norm.}} = 58.6 \pm 3.5 \text{ s}^{-1}$$

$$(k [\text{SOD}_1])_{\text{triso.}} = 82.8 \pm 6.5 \text{ s}^{-1}$$

Table 1
SOD activities of red cells in normal and trisomic 21 subjects.
The values shown represent $k [\text{SOD}_1]$. (See text.)

Normal (s^{-1})	Trisomic 21 (s^{-1})
57.8	72.9
57.7	83.4
56.0	90.9
63.4	90.0
60.0	85.3
54.5	74.6
50.7	74.5
65.7	91.0
61.8	—

giving the ratio:

$$(k [\text{SOD}_1])_{\text{triso.}} / (k [\text{SOD}_1])_{\text{norm.}} = 1.41 \pm 0.14$$

where 0.14, calculated for the same confidence, was obtained from Fieller's theorem.

4. Discussion

Numerous reports have recently demonstrated the biological role and importance of superoxide dis-

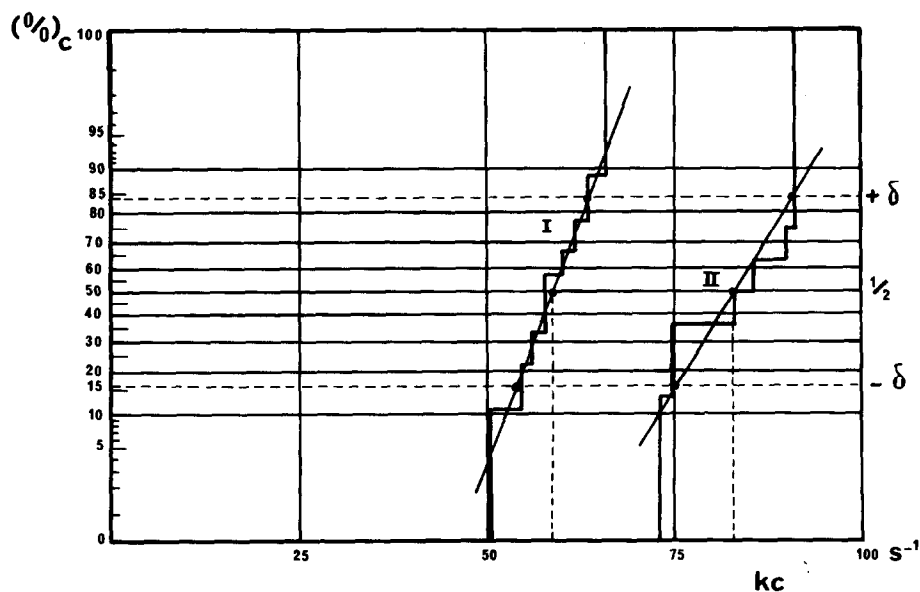


Fig. 3. Henry's diagram: (I) normal subjects. (II) Trisomic 21 subjects.

mutases. An accurate method of estimation of these enzymes might be of particular interest in some pathological conditions. Contrary to the other human cells, red cells which contain only one kind of SOD₁ cytosol superoxide dismutase, are thus a particularly suitable tissue for the estimation of this enzyme activity. A simple and rapid assay for SOD₁ in erythrocyte lysates without any purification would have been useful. Unfortunately the reaction of superoxide ions with the oxyhemoglobin-methemoglobin system [17] seems to interfere with the assay and our preliminary results have shown that removal of hemoglobin is necessary to obtain reproducible results.

Under these conditions, the reproducibility of the pulse radiolysis method was tested in determining k [SOD₁] twice for each sample no significant difference was observed. On the other hand, we observed no inactivation of the enzyme by H₂O₂, product of the catalysed reaction, by measuring k [SOD₁] after a second pulse on the same solution (H₂O₂ $\sim 2 \cdot 10^{-4}$ M).

The value of this ratio 1.41 is quite comparable to values obtained by other assay systems [3–5]. Otherwise, if we assume that there is no appreciable loss of SOD₁ in the preliminary treatment, we can calculate, using $k \sim 1.5 \times 10^9 \text{ M}^{-1} \cdot \text{s}^{-1}$ [18], average concentrations of SOD₁ contained in human erythrocytes as about 9 and 12.5×10^{-9} mol/g of hemoglobin for normal and trisomic 21 subjects respectively. The accuracy of these determinations and their significant difference, show that cytosol SOD₁ seems to be a constitutive enzyme whose rate of synthesis is not compensated in human red cells.

These observations seem to provide a better knowledge of enzyme synthesis mechanisms in mammalian cells and open a new field for research. They may also provide leads to therapy of chromosome abnormalities.

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