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# EFFECTS OF HYPERTHERMIC CONDITIONS ON THE REACTIVITY OF OXYGEN RADICALS

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Generation and reactivity of superoxide ($O_2^{\cdot -}$) and hydroxyl ($OH^{\cdot}$) radicals in enzymatic and radiolytic systems were investigated over the temperature range from 20°–50°C. The generation rate and reaction kinetics of both enzymatically and radiolytically produced superoxide radicals were determined by a cytochrome c reduction assay. For $OH^{\cdot}$ radical reaction studies the degradation of hyaluronic acid was assayed. An increase in temperature leads to a greater reactivity of both radicals, but in the case of an enzymatic source a disproportionate increase in the rate of generation is observed. In the pulse radiolysis system, the reactivity of superoxide radicals was found to be stimulated 15-fold over the temperature range from 20°C to 60°C, although the activity of superoxide dismutase was only minimally increased (about 1.6-fold). The results are discussed with respect to the possible importance of active oxygen species to the biological effects of hyperthermia.

Key words: Oxygen activation, Superoxide radicals, Hydroxyl radicals, Hyperthermia, Hyaluronic acid degradation

## INTRODUCTION

Within cells, various active oxygen species such as superoxide ($O_2^{\cdot -}$), hydrogen peroxide ($H_2O_2$) or hydroxyl radicals ($OH^{\cdot}$) are produced as reactive intermediates during metabolism[1]. These intermediates are either necessary components of metabolic reactions or inevitable side products. However, they are toxic and may damage the functional integrity of the cell, if the cellular control and protective mechanisms become insufficient[2,3]. It is well established that hyperthermia can produce regression of cancer in animal models and in humans[4]. The potential of hyperthermia as a combined treatment with radiation or chemotherapy for human cancer became more evident from recent clinical trials[5,6,7]. However, by studying

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*in-vitro* multiple targets like DNA, membranes or environmental factors, the cellular mechanism(s) of heat induced cytotoxicity[8], the synergistic interaction of some drugs with heat[9], and the relationship between temperature and maintenance of cellular integrity under thermal stress[10] still remain somewhat undefined at the molecular level.

With regards to active oxygen species, we proposed the concept that during hyperthermia the relationship between the production of potentially toxic species (e.g. oxygen radicals) and the protective mechanisms may deteriorate[11]. Regarding radiotherapy and chemotherapy, it has to be considered that most biological effects of radiation and many effects of antitumor antibiotics (e.g. adriamycin, bleomycin)[12,13,14] are due to the formation and action of oxygen radicals or other active oxygen species. In the present work, *in vitro* model reactions were used in order to study the effect of hyperthermic conditions on the generation and reactivity of such radicals.

## MATERIAL AND METHODS

The steady state radiolysis experiments were performed using a 50 kV Siemens Dermopan X-ray machine. The G-factors of the radicals of water radiolysis were taken as 2.7 ($OH^{\cdot}$) and 6.1 ($O_2^{\cdot -}$)[15]. The oxygen saturated formate system was used in order to convert all primary radicals of water radiolysis to superoxide radicals[15]. Radical concentrations were determined by chemical dosimetry[16].

Pulse radiolysis experiments were carried out with a Febetron 705 accelerator. The optical detection system (Osram xenon lamp XBO 450 W 4, Schoeffel monochromator, EMI 9659 Photomultiplier unit, Tektronix 7704 oscilloscope) has been especially designed for high resolution spectroscopic kinetic investigations between 200 and 850 nm. Rate constants, reaction orders and characteristics were analysed with a Wang 2200 computer. Superoxide radicals were produced by a short radiation pulse ($5 \times 10^{-8}$ s) with 1.8 MeV electrons in oxygen saturated aqueous solutions, which normally contained 0.1 M formate. In this system, the only radicals present 1 $\mu$s after the pulse were superoxide anions. Superoxide radical concentrations ($10^{-6}$ to $5 \times 10^{-5}$ M) were determined from the dose of the radiation pulse and the UV absorption of the radical[17].

The cytochrome c reduction assay was based on the method of McCord and Fridovich[18,19]. The radiolytic cytochrome c reduction assay was developed in our laboratory (manuscript in preparation). The amount of reduced cytochrome c was measured as the increase in absorbance at 550 nm. The experiments were carried out with thermostated cuvette holders at the indicated temperatures.

The overall catalytic activity of xanthine oxidase was determined by measuring the uric acid production at 293 nm according to the method of Kalckar[20] modified as described by Bergmeyer *et al.*[21].

For the hyaluronic acid degradation experiments, oxygen radicals were produced by three different methods: radiolysis, xanthine oxidase reaction and autoxidation of 6,7-dimethyl-5,6,7,8-tetrahydropterin. Hyaluronic acid degradation was measured as percentage decrease in initial viscosity[22]. The viscosity determinations were carried out using a Schott Micro-Ubbelohde viscometer in a precisely temperature controlled water bath. The influence of the individual test compound on the stability of the hyaluronic acid specific viscosity was assessed by control experiments. For each test

substance, the viscosity determinations were also carried out in the absence of hyaluronic acid. The results are expressed as the percentage decrease in hyaluronic acid viscosity due to a particular substance or reaction. Each percentage value is the mean of at least three determinations.

Cytochrome c and xanthine oxidase were obtained from Boehringer, Mannheim, Cu/Zn superoxide dismutase was from Diagnostic Data, California, hyaluronic acid from Serva, Heidelberg and 6,7-dimethyl-5,6,7,8-tetrahydropterin from Sigma, Munich.



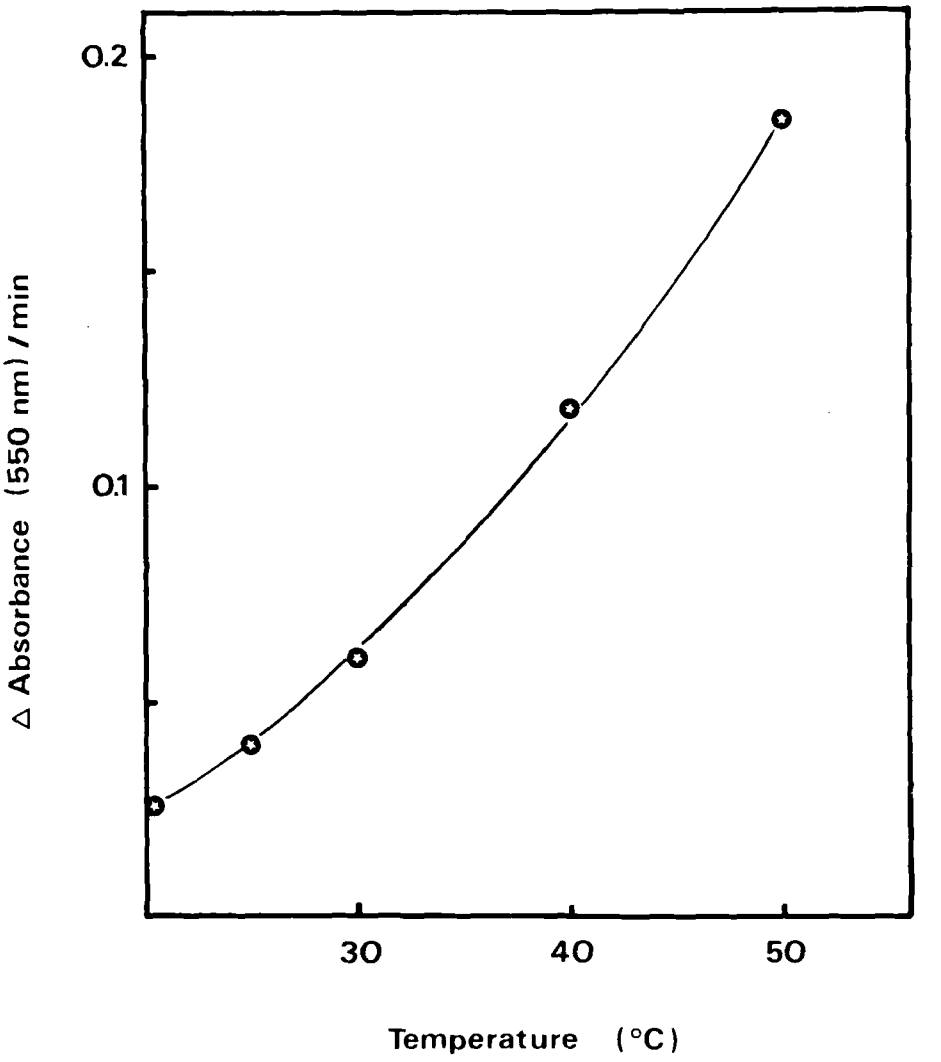


FIGURE 1 Effect of temperature on the yield of reduced cytochrome c by xanthine/xanthine oxidase. The air saturated mixture contained in 2 ml: phosphate buffer 33 mM pH 7.8, cytochrome c $4.2 \times 10^{-5}$ M, xanthine $5.6 \times 10^{-4}$ M, xanthine oxidase 75 $\mu$g. The reaction time was 60 sec.

## RESULTS

### *Reaction of superoxide radicals with cytochrome c at different temperatures*

The reduction of cytochrome c by superoxide radicals produced by the xanthine/xanthine oxidase reaction is a well known reaction[18]. Figure 1 shows the effect of a temperature rise from 20°C to 50°C on the amount of cytochrome c being reduced under these conditions. The individual rate of reduction at 20, 30, 40 and 50°C was virtually constant over a reaction time of 2.5 min (data not shown). The relative increase in cytochrome c reduction with increasing temperature is shown in Fig. 1. For example, by comparison of the rates observed at 37° and 44°C we calculated an increase in the yield of the superoxide radical dependent reaction product of about 43%. This effect of temperature was due to an enhanced formation of superoxide radicals by the xanthine/xanthine oxidase reaction. In the presence of superoxide dismutase ($10^{-6}$ M) added to the cuvette prior to the addition of xanthine oxidase the reduction of cytochrome c was completely inhibited at 20°–50°C (data not

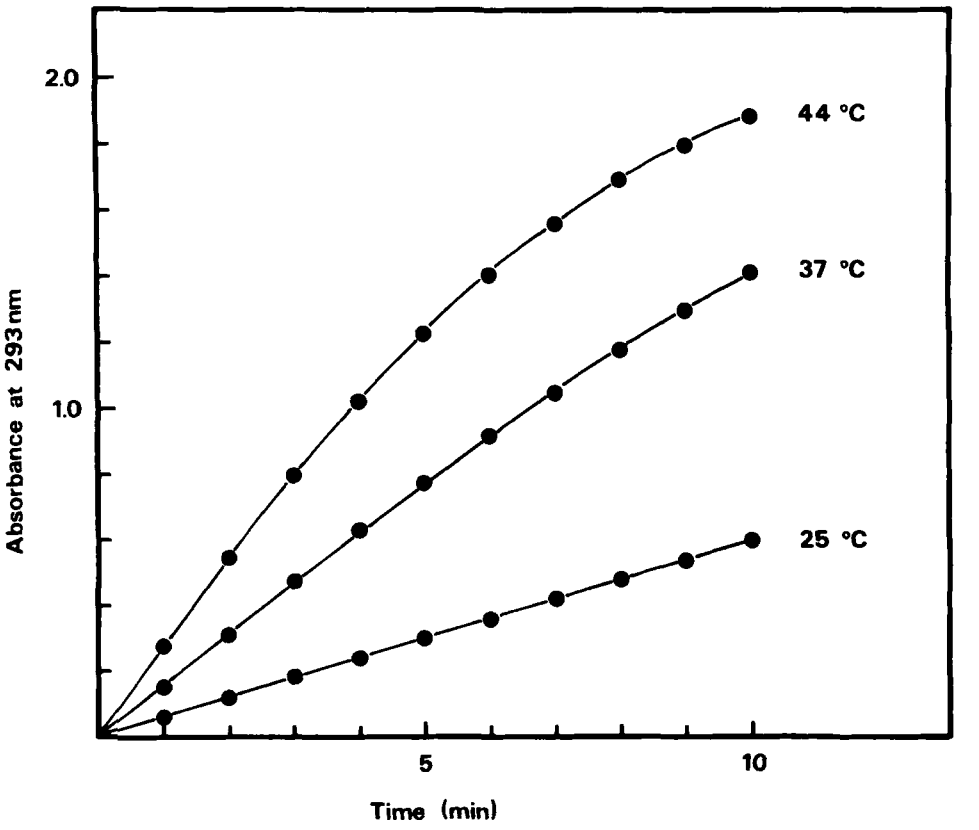


FIGURE 2 Uric acid production by the xanthine/xanthine oxidase reaction at different temperatures. The reaction mixture was saturated with oxygen and contained in 3 ml: phosphate buffer 50 mM, pH 7.4, EDTA 0.1 mM, xanthine 0.2 mM and xanthine oxidase 50 μg. The uric acid production was monitored at 293 nm.

shown) whereas inactivated superoxide dismutase had no effect. Inactivation of superoxide dismutase was accomplished by acidifying the protein solution to pH 2.0 followed by dialysing in order to remove the metal from the active side of the enzyme. However, after 30 min of heat treatment of superoxide dismutase, only a little decrease in enzyme activity was observed. In order to demonstrate that the increase in $O_2^-$ production is only due to an increased turnover rate of xanthine oxidase under hyperthermic conditions and not due to other reactions, uric acid production over the same range of temperature was also determined. The results of the temperature dependent increase of uric acid formation by xanthine/xanthine oxidase reaction are shown in Fig. 2. The increased amount of uric acid observed at 44°C compared to the amount at 37°C allows stoichiometric correlation with the amount of cytochrome c reduced by this reaction under comparable conditions.

Figure 3 shows the effect on cytochrome c reduction, when the superoxide radicals were produced by radiation at a rate of $2 \times 10^{-6}$ M/min. Interestingly, increasing the temperature from 20°C to 44°C *decreased* cytochrome c reduction by about 10% after 10 min reaction time. The inhibitory effect of superoxide dismutase indicated that the reduction of cytochrome c under the experimental conditions was due to superoxide radicals. If the radiation dose was lowered resulting in $3 \times 10^{-7}$ M

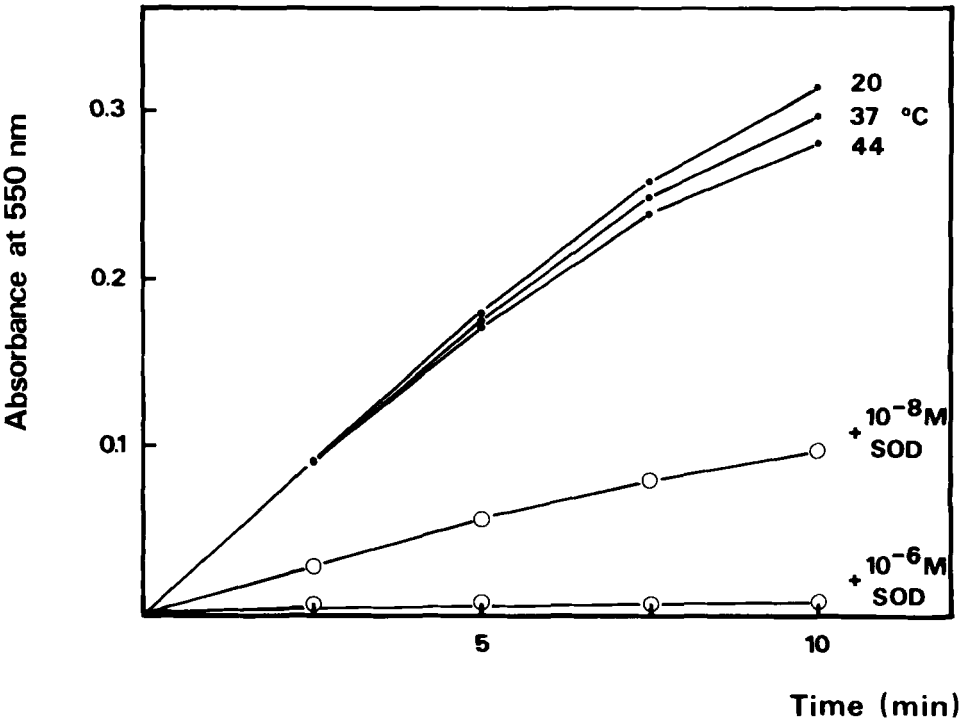


FIGURE 3 Cytochrome c reduction by a steady state radiolysis system. The superoxide radical generation rate was $2 \times 10^{-6}$ M/min. The reaction mixture contained in 3 ml: phosphate buffer 66 mM, pH 7.8, sodium formate $10^{-2}$ M, cytochrome c $2 \times 10^{-5}$ M. The results with superoxide dismutase at 20°C, 37°C and 44°C were statistically the same at each concentration of the enzyme ($10^{-8}$ M and $10^{-6}$ M) and lay within the area of the open circles. The reaction mixture was saturated with oxygen.

superoxide radicals being formed per minute, cytochrome c reduction was found to be increased again at higher temperatures (37°C and 44°C) (Fig. 4).

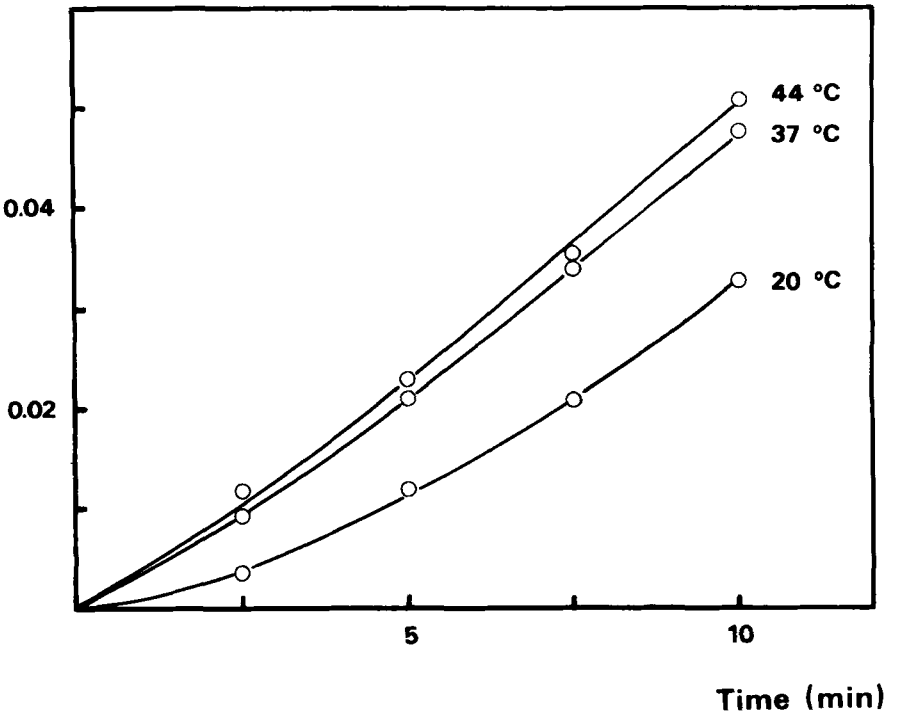


FIGURE 4 Cytochrome c reduction by a steady state radiolysis system. The superoxide radical generation rate was $3 \times 10^{-7}$ M/min. The reaction mixture contained in 3 ml: phosphate buffer 66 mM, pH 7.8, sodium formate $10^{-2}$ M, cytochrome c $10^{-4}$ M. The reaction mixture was saturated with oxygen.

*Determination of rate constants of superoxide radical reactions by pulse radiolysis*

For the study of the reaction kinetics and rate constants of the superoxide radical, pulse radiolysis combined with kinetic spectroscopy is the most versatile method, because the radical itself can be monitored by its UV absorption at 250 nm with a time resolution of less than one microsecond[23]. In the presence of superoxide dismutase for example, the life time of superoxide radicals is only some tens of microseconds. Table I shows the reaction rate constants of superoxide radicals with each other (spontaneous dismutation) or with Cu/Zn superoxide dismutase at 20°C to 60°C. The temperature of 60°C was chosen in order to see a possible heat dependent instability of the enzyme and a more pronounced effect with the radical-radical reaction. The rate of spontaneous dismutation of superoxide (radical-radical reaction) at 60°C was found to be about fifteen times higher than that found at 20°C. The rate of reaction of the $O_2^-$ with Cu/Zn superoxide dismutase was only increased by a factor of about 1.6, when the temperature was increased from 20°C to 60°C. After heating of the enzyme for 75 min there was virtually no loss of activity. The additional presence of EDTA at a high concentration ($10^{-3}$ M) at 60°C also did not alter the enzymatic activity significantly.

TABLE I
Rate constants of superoxide radical reactions at 20°C and 60°C

| System | Temperature °C | k ($\ell mol^{-1}s^{-1}$) |
|---|---|---|
| $O_2^{\cdot-}$ + $O_2^{\cdot-}$ (spontaneous dismutation) | 20 | $1.8 \pm 0.4 \times 10^5$ |
| | 60 | $3.0 \pm 0.6 \times 10^6$ |
| $O_2^{\cdot-}$ + SOD ($9.1 \times 10^{-6}$ M) | 20 | $2.1 \pm 0.1 \times 10^9$ |
| 5 min. | 60 | $3.4 \pm 0.2 \times 10^9$ |
| 75 min. | 60 | $3.3 \pm 0.3 \times 10^9$ |
| $O_2^{\cdot-}$ + SOD + EDTA ($10^{-3}$ M) 15 min. | 60 | $3.1 \pm 0.2 \times 10^9$ |

Rate constants were determined by pulse radiolysis and regression analysis of the decay kinetics of superoxide radicals at pH 7.2, monitored at 250 nm. The reaction mixtures were kept for the time indicated at the respective temperature, before the measurements were carried out. The concentration of superoxide radicals, produced by the electron pulse, was $10^{-5}$ M.
SOD: superoxide dismutase,
EDTA: ethylene diamine tetraacetic acid

These results therefore indicate that the copper in the active site of superoxide dismutase could not be removed by EDTA even under these high temperature conditions which is also a demonstration of the unusual stability of superoxide dismutase.

*Hyaluronic acid degradation by hydroxyl radicals at different temperatures*

Among the active oxygen species only hydroxyl radicals are known to degrade hyaluronic acid[24]. Hyaluronic acid is not degraded by reducing radicals such as hydrated electrons, hydrogen atoms or $^{\cdot}COO^-$, nor by superoxide radicals or hydrogen peroxide[24]. When radiation was used as the source of hydroxyl radicals, only a minor increase in degrading activity was observed at higher temperatures (Fig. 5). After a period of about 4 min at the end of irradiation, the viscosity of hyaluronic acid remained constant at different temperatures (20°, 37° and 44°C).

However, in the presence of formate ($5 \times 10^{-1}$ M) as an effective $OH^{\cdot}$ radical scavenger no hyaluronic acid degradation at all was observed (data not shown). Using the xanthine/xanthine oxidase reaction for the production of hydroxyl radicals[25], a marked effect over the temperature range studied was observed (Fig. 6). Increasing the temperature from 37°C to 44°C a quite remarkable additional hyaluronic acid degrading effect took place which is attributed to a temperature dependent stimulation of hydroxyl radical production by the xanthine/xanthine oxidase reaction. 6,7-Dimethyltetrahydropterin, which easily undergoes autoxidation, has been shown to give rise to the formation of a highly oxidizing species[26]. During autoxidation of 6,7-dimethyletrahydropterin a hyaluronic acid degrading activity was observed. This system was also studied at different temperatures and the results are shown in Fig. 7. This observation is of particular interest, because the formation of an $OH^{\cdot}$ radical-like oxidative species by this pterin was described to be dependent upon the presence of chelated iron[27].

Using radiolysis as well as the xanthine/xanthine oxidase reaction, formate was found to be the most efficient inhibitor of $OH^{\cdot}$-mediated hyaluronic acid degradation as previously demonstrated[24]. The addition of increasing concentrations of formate to

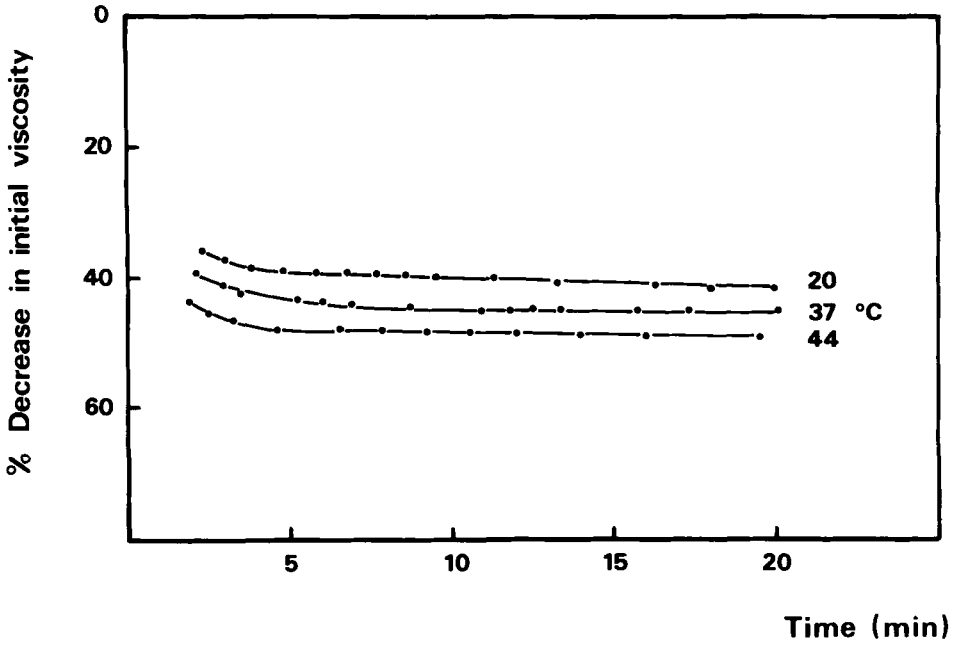


FIGURE 5 Hyaluronic acid degradation by radiolytically produced hydroxyl radicals. The generation rate was $10^{-6}$ M/min, the figure shows the subsequent reaction after the end of 4.5 min irradiation. The reaction mixture was saturated with oxygen and contained in 3 ml: phosphate buffer 66 mM, pH 7.4, hyaluronic acid 2 mg.

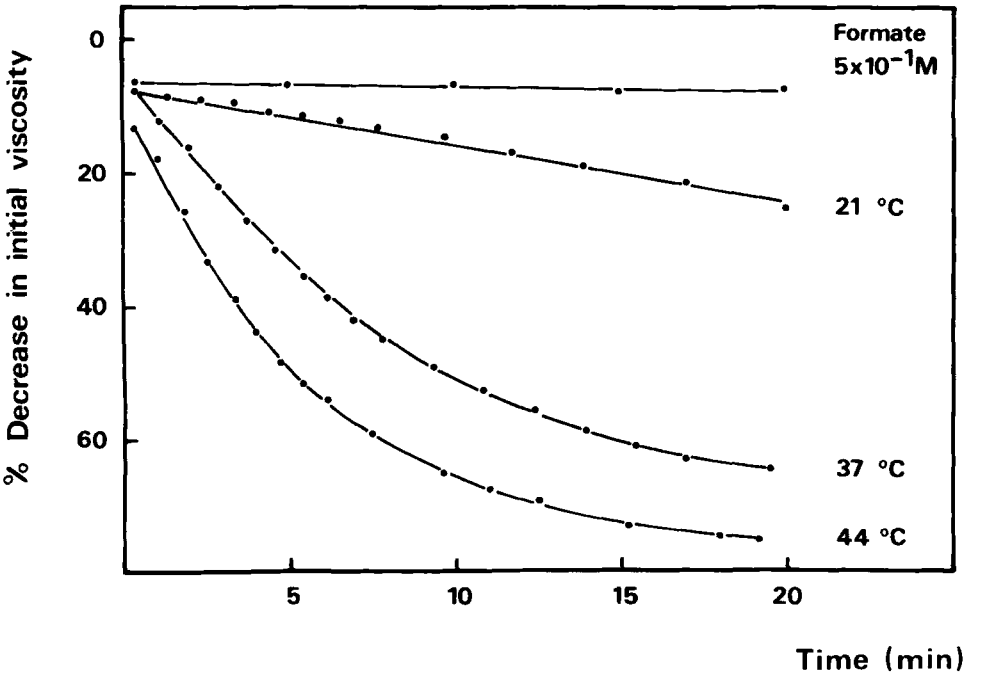


FIGURE 6 Hyaluronic acid degradation by xanthine/xanthine oxidase. The reaction mixture was saturated with oxygen and contained in 3 ml: phosphate buffer 66 mM, pH 7.4, xanthine 0.6 mM, xanthine oxidase 150 $\mu$g, hyaluronic acid 2 mg and where indicated formate 5 × $10^{-1}$ M. The step of decrease in viscosity at zero time was due to dilution by addition of the volume containing xanthine oxidase to start the reaction.

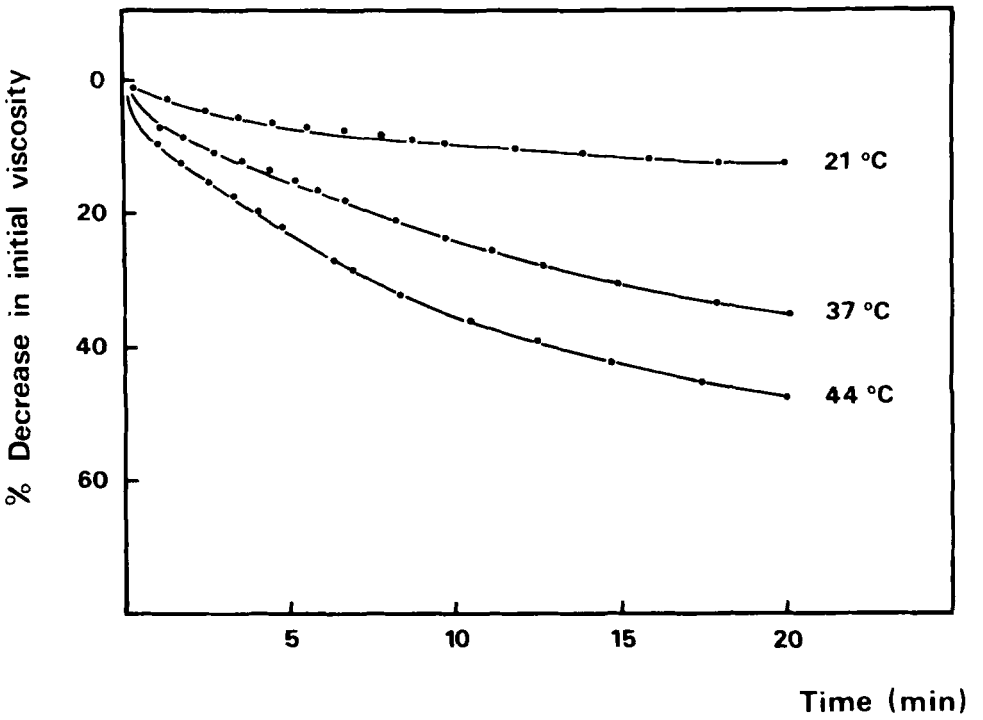


FIGURE 7 Hyaluronic acid degradation by autoxidizing 6,7-dimethyltetrahydropterin. The reaction mixture was saturated with oxygen and contained in 3 ml: phosphate buffer 66 mM, pH 7.4, hyaluronic acid 2 mg, 6,7-dimethyltetrahydropterin 140 $\mu$M.

these systems reduced hyaluronic acid degradation. Complete inhibition by formate was found at a concentration of 0.5 M[24]. Hyaluronic acid degradation caused by 6,7-dimethyltetrahydropterin was fully inhibited by the presence of $2 \times 10^{-3}$ M formate at 37°C (data not shown).

## DISCUSSION

The effect of temperature on cytochrome c reduction by superoxide radicals was found to be strictly dependent on the radical source. It is well known from radiation chemistry that the production yield of radiolytically generated radicals is almost constant over the temperature range from 20°C to 50°C. Therefore the temperature dependency of cytochrome c reduction using the xanthine/xanthine oxidase reaction has to be attributed to the superoxide producing activity of this enzyme, where a small increase in temperature resulted in a large enhancement of the radical generation rate. Hyperthermic conditions at 44°C as compared to our results at 37°C increased the production of superoxide radicals by this enzyme to about 43% due to an increased turnover rate. In order to explain the paradoxical effect shown in Fig. 3, where an increase in temperature led to a decrease in cytochrome c reduction, competing reactions have in these cases to be considered. Superoxide radicals react with cytochrome c, but also with each other.

$$O_2^{\cdot -} + O_2^{\cdot -} \xrightarrow{+2H^+} H_2O_2 + O_2 \quad (K_1)$$

$$\text{Cyt c}_{(\text{oxid})} + O_2^{\cdot -} \longrightarrow \text{Cyt c}_{(\text{red})} + O_2 \quad (K_2)$$

Whilst at room temperature, the reaction rate constants $K_1$ and $K_2$ are of the same order of magnitude (about $10^5$ $\ell mol^{-1}.s^{-1}$) at higher temperatures, $K_1$ has to be assumed to be greater than $K_2$. In fact, the pulse radiolysis results showed that the reactivity of superoxide radicals is markedly increased by a rise in temperature. This effect is certainly not restricted to radical-radical reactions, but probably applies also to reactions of superoxide radicals with many cellular components and with different slopes of temperature dependent reactivity.

Although superoxide radicals themselves are considered to be of moderate toxicity[28], the increased reactivity during hyperthermia may be of particular importance. The potential danger of superoxide radicals lies in their ability to lead to the formation of hydroxyl radicals[1,29,30,31], which are highly reactive species. If at higher temperatures $K_1$ is much greater than $K_2$, it would be expected that more superoxide radicals disappeared by radical-radical reactions and less by reaction with cytochrome c, as is in fact shown in Fig. 3. This is supported by the data of Fig. 4, where cytrochrome c was present in excess compared to the amount of superoxide radicals being generated. On the basis of these considerations, it can be concluded from the data in Fig. 1 that the generation rate of superoxide radicals by the xanthine/xanthine oxidase reaction at higher temperatures must be increased to such an extent that the increased rate of radical-radical reaction is insignificant.

Whilst under hyperthermic conditions the reactivity of superoxide radicals and also its biochemical production rate was increased, the rate of superoxide radical elimination by superoxide dismutase remained almost unaltered (Table I). This means that hyperthermia may lead to a relative loss of cellular protective capacity with respect to this enzyme. On the other hand, superoxide dismutase turned out to be stable also at temperatures above that used in hyperthermia treatments (40–43°). The stability of this copper containing enzyme usually isolated from erythrocytes remained constant even in the presence of high concentrations of the metal chelator EDTA. In contrast, Cu/Zn superoxide dismutase isolated from spleen of patients suffering from malignant lymphoma was found to be more altered in its specific activity by treatment with EDTA compared to the enzyme isolated from normal human spleen[32]. More general, the activity of superoxide dismutase has been found to be diminished in most malignant tissues[33]. Also the copper containing superoxide dismutase appeared to be slightly lower in 31 different malignant cell lines as compared to normal tissues[34]. Supporting the idea that the activity of this enzyme might be involved in a protective mechanism against thermosensitization, cells treated with diethyldithiocarbamate, a strong inhibitor of Cu/Zn superoxide dismutase, were found to be significantly more sensitive to heat than untreated cells[35]. More data are however needed to evaluate the possible role of this enzyme in the mechanism of heat damage due to its intrinsic properties or its structure and function.

The observed hydroxyl radical dependent hyaluronic acid degradation can also be interpreted on the basis of reaction kinetics. At a constant (radiolytic) generation rate over the temperature range studied here, due to the excess concentration of hyaluronic acid the reaction between hyaluronic acid and $OH^{\cdot}$ was much favoured over the reaction between two hydroxyl radicals, which would result in the formation of hydrogen peroxide. The rate of formation of hydroxyl radicals by the enzymatic system was markedly enhanced by increasing the temperature. Under hyperthermic conditions,

autoxidation of 6,7-dimethyltetrahydropterin also resulted in an increased formation of hyaluronic acid degrading species, which can be assumed to be a hydroxyl radical like species[24,26]. This reaction is not yet understood in detail, but may be of importance, because pterins are components of folic acid and therefore essential in cell metabolism.

In contrast to these relatively simple model reactions, in a cellular system a great number of radical reactions are in a complex equilibrium and also under control of enzymatic and other non-enzymatic protective systems. Interestingly, in support of the concept described above oxygen radicals were found to play an essential role in the mechanism of thermosensitization of Chinese hamster ovary cells by thiol compounds[30]. The generation of superoxide radicals and hydrogen peroxide by thiols was found to be strongly dependent on environmental conditions such as pH, metal ions, temperature, and the concentrations of the thiol compounds themselves[36]. In conclusion, under hyperthermic conditions, radical reactions of active oxygen species could gain importance over other reactions, as was found in the present study. This could result in an increased toxicity for the cell.

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