Hydrogen peroxide production during experimental protein glycation

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The accumulation of hydrogen peroxide (H_2O_2) during incubations of protein with glucose (experimental glycation) has previously been too low for direct measurement although it is suggested to be the precursor of protein-damaging hydroxylating agents. We have thus developed a simple H_2O_2-measuring technique which relies upon the rapid peroxide-mediated oxidation of Fe^{2+} to Fe^{3+} (catalysed by sorbitol) under acidic conditions followed by reaction of the latter cation with the dye, xylenol orange. We have used the method to demonstrate that incubation mixtures of protein and glucose generates nanomolar levels of hydrogen peroxide in the presence of protein under physiological conditions of pH and temperature.

Hydrogen peroxide; Xylenol orange; Glucose autoxidation; Protein glycation

1. INTRODUCTION

The exposure of proteins to glucose in vitro (experimental protein glycation) is widely used as a model for tissue damage associated with diabetes mellitus and ageing. Recently, however, it has been suggested that transition metal-catalysed glucose enediol oxidation (glucose 'autoxidation') is the factor responsible for protein damage occurring during in vitro experimental glycation rather than the addition of monosaccharide to protein amino groups per se [1,2]. While there is little doubt that free radicals and transition metals appear to be involved in alterations to protein exposed to glucose in vitro the production of H_2O_2 by glucose in the presence of protein has only been inferred, by the partial protective effect of catalase [2], since the steady-state levels of H_2O_2 are in the submicromolar range and thus appear to be too low for direct detection by most methods [1]. Such inferential implication of H_2O_2 production is obviously unsatisfactory. We have thus developed a sensitive peroxide-measuring technique to show that incubation mixtures containing glucose and protein accumulate nanomolar quantities of H_2O_2 under physiological conditions.

2. EXPERIMENTAL

Xylenol orange, ammonium ferrous sulphate, hydrogen peroxide, cumene peroxide, butyl peroxide, sorbitol, sucrose, glucose, fructose, formic acid, dimethylsulphoxide, and catalase (Sigma type C-40) were all obtained from Sigma (Poole, Dorset) or Aldrich (Poole, Dorset) and were of the highest purity available. Albumin (Bovine: Fraktion V) was obtained from Boehringer (Mannheim, FRG). Absorbance data were gathered on a Pye Unicam Series 8700 series UV/VIS spectrophotometer. The concentration of H_2O_2 in stock solutions was calculated using the extinction coefficient of 43.6 $M^{-1}\cdot cm^{-1}$ at 240 nm [3]. Concentrations of cumene peroxide and butyl peroxide were taken as given.

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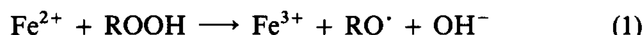
3. RESULTS AND DISCUSSION

3.1. *The use of xylenol orange to detect peroxide-generated Fe^{3+}*

Ferrous ion (Fe^{2+}) is relatively stable to autoxidation in dilute acid, but can be oxidised by peroxides to yield Fe^{3+} and hydroxyl/alkoxyl radicals in a well-known (but simplified) reaction which proceeds rapidly at room temperature [4].



$$Fe^{2+} + ROOH \longrightarrow Fe^{3+} + RO^{\cdot} + OH^{-} \quad (1)$$

Detection of Fe^{3+} ions formed in a reaction mixture consisting of ferrous sulphate in dilute H_2SO_4 on addition of peroxide can be achieved using the dye xylenol orange, which binds Fe^{3+} forming a complex which absorbs strongly between 540 and 580 nm in dilute acid [5,6]. The extinction coefficient of the Fe^{3+}-xylenol orange complex at 560 nm (the absorbance maximum) was determined using freshly-made solutions of ferric chloride and determined to be 1.5×10^4 $M^{-1}\cdot cm^{-1}$ in 25 mM H_2SO_4 at room temperature.

In the course of experiments designed to measure recovery of H_2O_2 in protein/sugar incubation mixtures using a peroxide assay system consisting of ammonium ferrous sulphate (250 μM), H_2SO_4 (25 mM) and xylenol orange (100 μM) [7] we observed that certain substances, notably fructose, sorbitol, sucrose, glucose and formic acid (all at a concentration of 100 mM at which concentration the effect was maximal) produced very high enhancements of colour yield relative to the unmodified system (Fig. 1). Fructose gave a particular-

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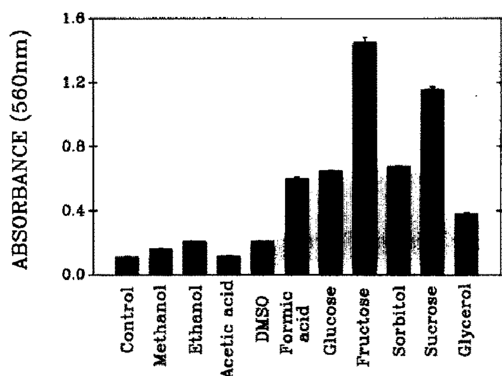

Fig. 1. The reaction of H_2O_2 (2 μM) with 100 μM xylenol orange and 250 μM Fe^{2+} in 25 mM H_2SO_4 in the presence of various compounds (100 mM). Absorbance was read at 560 nm after 45 min at room temperature. Data are the mean ± SD of duplicate determinations.

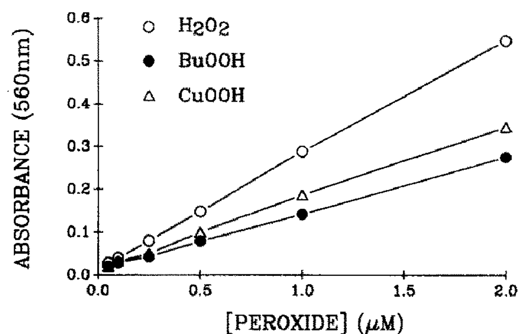

Fig. 2. Standard curves of H_2O_2, *t*-butyl peroxide and cumene peroxide in the range 0–2 μM. Peroxides were measured with 100 μM xylenol orange, 250 μM Fe^{2+} and 100 mM sorbitol in 25 mM H_2SO_4. Absorbance was read at 560 nm after 45 min at room temperature. Data are the mean ± SD of duplicate determinations.

ly high colour enhancement but the signal was unfortunately unstable. In the case of sorbitol, however, almost the same signal intensity was observed when the samples had been allowed to stand overnight at room temperature.

3.2. *Sensitivity of the assay*

Standard curves of H_2O_2, *t*-butyl peroxide (BuOOH) and cumene peroxide (CuOOH) in an assay system consisting of 250 μM ammonium ferrous sulphate, 100 μM xylenol orange, 25 mM H_2SO_4 and 100 mM sorbitol are shown in Fig. 2. We observed that the peroxides were reactive in the order H_2O_2 > CuOOH > BuOOH. The extinction coefficients (estimated by curve fitting to a first-degree function) were $2.67 \times 10^5\ M^{-1} \cdot cm^{-1}$ for H_2O_2; for cumene peroxide $1.67 \times 10^5\ M^{-1} \cdot cm^{-1}$ and for butyl peroxide $1.27 \times 10^5\ M^{-1} \cdot cm^{-1}$. Given that the extinction coefficient of the Fe^{3+}-xylenol orange complex is $1.5 \times 10^4\ M^{-1} \cdot cm^{-1}$, it follows that approximately 18 mol of Fe^{2+} must be oxidised to Fe^{3+} for each mol of H_2O_2 in the presence of sorbitol and somewhat less for the other peroxides. This indicates the presence of substantial chain oxidation giving the assay a sensitivity of 25–50 nM peroxide, assuming the availability of a spectrophotometer which can register absorbance changes of 0.01 AU. The reaction is oxygen-dependent (not shown) and is presumably dependent on the ability of sorbitol to scavenge hydroxyl radicals (produced in Eqn 1) to yield peroxyl radicals [8,9] which would propagate Fe^{2+} oxidation.

3.3. *Glucose autoxidation*

Using the assay described here, the accumulation of H_2O_2 with respect to time and concentration in glucose and glucose/protein mixtures can be monitored (Fig. 3). Catalase completely abolished colour development when added prior to the peroxide reagent demonstrating that H_2O_2 was being measured. The presence of albumin (100 μg/ml) in the peroxide-measuring reaction mixture caused no loss of H_2O_2 recovery (not shown) and levels of H_2O_2 calculated agreed well with that estimated using the extinction coefficient above, or derived from spiking identical glucose/protein samples with small concentrations of H_2O_2.

In the presence of serum albumin (1 mg/ml) the steady-state levels of H_2O_2 detected were approximately 6-fold lower than in the absence of the protein. Albumin chelates copper ion [10] and thus may inhibit glucose 'autoxidation' via chelation of trace amounts of copper since autoxidation is dependent upon transition metal [2]. Reaction of the open-chain form of

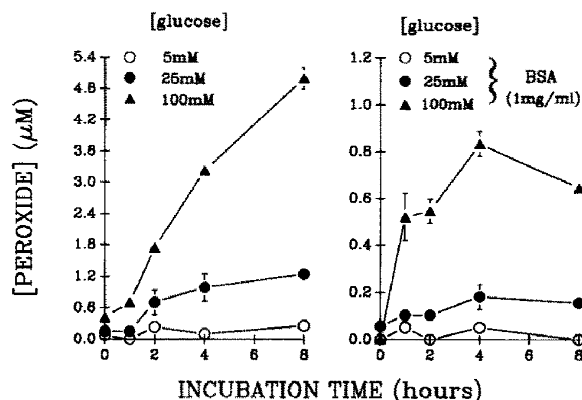

Fig. 3. The production of H_2O_2 by autoxidising glucose. Glucose (5 mM, 25 mM and 100 mM) with or without 1 mg/ml bovine serum albumin was incubated in potassium phosphate buffer (pH 7.4, 10 mM) at 37°C. After incubation, 100 μl samples were added to 900 μl of reagent (100 μM xylenol orange, 250 μM Fe^{2+} and 100 mM sorbitol in 25 mM H_2SO_4). Absorbance was read at 560 nm after 45 min incubation at room temperature following a 2 min centrifugation at 1.000 × *g* to remove any flocculated protein. Values (peroxide concentration in the sample) are the mean ± SD obtained from duplicate assays. Catalase (Sigma type C-40) added to the sample (at a concentration of 100 U/ml) prior to addition of reagent abolished the colour development.

glucose (which is the precursor of glucose enolisation and autoxidation [1,2]) with protein amino groups may also retard the formation of H_2O_2. Although the steady-state concentrations of H_2O_2 are low in absolute terms they are in the range expected to contribute to protein damage. At 25 mM glucose a steady-state level of 200 nM H_2O_2 is achieved (compared with a protein concentration of 15 μM) and this is also in the concentration range previously observed for production of hydroxylating agents [2] which are the proximal agents of protein oxidation. In experimental systems of glycation in which it is desired to examine solely the structural/functional effects of the addition of glucose to protein amino groups, rather than central or peripheral oxidative events, metal-chelating agents and/or catalase should be included to inhibit the formation/accumulation of H_2O_2. Consistent with this we have recently shown that glucose stimulates the peroxidation of the lipid moiety of low density lipoprotein but this is inhibited by the metal chelating agent diethylenetriaminepenta-acetic acid [11].

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