Kinetic study of the oxidation mechanism of glutathione by hydrogen peroxide in neutral aqueous medium

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The oxidation kinetics of glutathione (GSH) by hydrogen peroxide has been studied at neutral pH for different concentration ratios $[GSH]_0/[H_2O_2]_0$ between 0.2 and 2 ($5 \times 10^{-4}\ M \leq [H_2O_2]_0 \leq 2.5 \times 10^{-3}\ M$; $4 \times 10^{-4}\ M \leq [GSH]_0 \leq 2.5 \times 10^{-3}\ M$). In all cases studied, glutathione disulfide GSSG is the main product formed via two different oxidation ways, each of them contributing respectively to 80–85% and 10–15%.

Our kinetic data indicate that an important fraction of hydrogen peroxide disappears without oxidizing the thiol function. This can be attributed to a combination between GSH and H_2O_2 protecting the sulfide group. Chemical evidences of the existence of a peroxide bond with GSSG are described. In our experimental conditions, the overall oxidation equation is 2 mol GSH reacting with 2 mol H_2O_2 giving 1 mol GSSG. It is very different from the usually accepted stoichiometric reaction:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

A kinetic scheme is proposed and the corresponding rate constants are determined.

Key words: glutathione, hydrogen peroxide, kinetic mechanism, glutathione disulfide.

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Opérant à un pH neutre et à des rapports de concentration de $[GSH]_0/[H_2O_2]_0$ allant de 0,2 à 2 ($5 \times 10^{-4}\ M \leq [H_2O_2]_0 \leq 2{,}5 \times 10^{-3}\ M$; $4 \times 10^{-4}\ M \leq [GSH]_0 \leq 2{,}5 \times 10^{-3}\ M$), on a étudié la cinétique de l'oxydation du glutathion (GSH) par le peroxyde d'hydrogène. Dans tous les cas étudiés, le disulfure de glutathion, GSSG, est le produit principal qui est formé par deux voies d'oxydation principales qui apportent des contributions respectives de 80 à 85% et 10 à 15%.

Nos données cinétiques indiquent qu'une fraction importante du peroxyde d'hydrogène disparaît sans oxyder la fonction thiol. On peut attribuer ce fait à une combinaison du GSH et du H_2O_2 qui protégerait le groupement sulfure. On décrit des données chimiques qui suggèrent l'existence d'une liaison du peroxyde avec le GSSG. Dans nos conditions expérimentales, l'équation globale d'oxydation implique donc deux moles de GSH qui réagissent avec deux moles de H_2O_2 pour donner une mole de GSSG. Cette équation diffère beaucoup de la réaction stoechimétrique généralement admise:

$$H_2O_2 + 2GSH \rightarrow GSSG + 2H_2O$$

On propose une schéma cinétique et on a déterminé les constantes de vitesse correspondantes.

Mots clés : glutathion, peroxyde d'hydrogène, mécanisme cinétique, disulfure de glutathion.

[Traduit par la revue]

Introduction

Glutathione (GSH) is present in almost all cells in rather high concentration. The conversion of glutathione (GSH) to glutathione disulfide GSSG, *in vivo*, is catalyzed by glutathione peroxidase, which acts on hydrogen peroxide and other peroxides. The overall oxidation reaction of GSH by H_2O_2 in presence of peroxidase is

$$[1]\quad 2GSH + H_2O_2 \xrightarrow{GPX} GSSG + 2H_2O$$

It is generally agreed that in the absence of the enzyme the overall oxidation process is not different from this reaction (1). But the real mechanism of oxidation of GSH by H_2O_2 is unknown.

However, the oxidation kinetics of a variety of sulfur compounds such as hydrogen sulfide (2), sulfur dioxide (3), thioureas (4), thioxane (5), thiocyanate (6, 7) and 2 mercaptoethanol (8) by hydrogen peroxide in aqueous solution have been studied. A common mechanism for the acid-catalyzed nucleophilic attack consists of a heterolytic cleavage of oxygen–oxygen bond of hydrogen peroxide with the release of either hydroxide ion or water as leaving group according to

$$RSH + H_2O_2 \rightarrow RSOH + H_2O$$

The hydroxy intermediate (RSOH) subsequently reacts with either a second molecule of reductant to form a dimer as a final product or with an additional molecule of H_2O_2 (2).

Concerning the reaction of H_2O_2 with GSH, McCormic *et al.* (9) have shown that a UV chromophore having a maximal absorption at 305 nm appears during the reaction. But the kinetics of the reaction were not studied. The same observation was obtained when aerated aqueous solutions of GSH were irradiated with a 254 nm radiation (9). From several comparative studies with other thiols in the same conditions, they attributed the absorbance at 305 nm to a product which is formed on the *N*-acetylcysteinamide (—CONH—CH(—CH_2—SH)—$CONH_2$) part of GSH.

In order to carefully investigate the mechanism of oxidation of GSH by H_2O_2, the kinetics of this reaction have been studied in neutral aerated aqueous medium, in absence of peroxidase for different concentrations: $4 \times 10^{-4}\ M \leq [GSH]_0 \leq 2.5 \times 10^{-3}\ M$, $5 \times 10^{-4}\ M \leq [H_2O_2]_0 \leq 2.5 \times 10^{-3}\ M$, and different ratios $[GSH]_0/[H_2O_2]_0$ (0.2 to 2).

Materials and methods

Chemicals were of the purest grade available and were used without further purification. The only exception was glutathione sulfonic acid which was used as a standard for HPLC and spectral analysis. It was prepared according to Calam and Waley (10). GSH and GSSG were obtained from FLUKA; 5,5′-dithiobis(2-nitrobenzoic acid), NADPH, glutathione reductase were supplied by Sigma Chemical Co.; hydrogen

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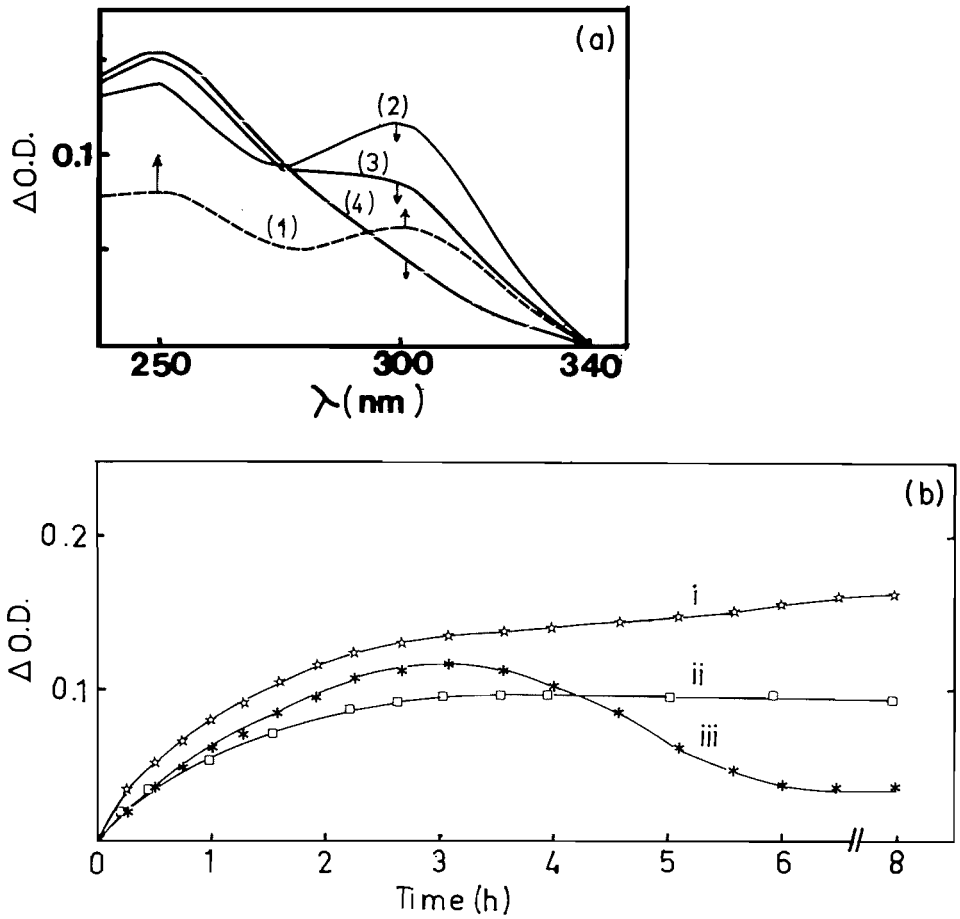


FIG. 1. (*a*) Differential absorption spectra at various times after mixing. $[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ *M*, phosphate buffer 0.1 *M*, 22°C, pH 7, *l* = 0.875 cm. Reference: $[GSG]_0 = 2 \times 10^{-3}$ *M* (*l* = 0.4375 cm) (two chambers cell, see Experimental section). (*1*) 1 h, (*2*) 3 h 5 min, (*3*) 4 h 35 min, (*4*) 5 h 35 min after mixing of the reactants. (*b*) Evolutions of the differential absorption densities as a function of time at three different wavelengths: (☆) λ 250 nm, (□) λ 268 nm, (★) λ 305 nm.

peroxide, sodium acid phosphate were from Merck. All solutions were prepared with triply distilled water, the purity of which was controlled by conductivity measurements (10^{-6} Ω cm^{-1}).

Thiol concentration was determined with Ellman's reagent, 5,5'-dithiobis(2-nitrobenzoïc acid) which undergoes a thiolate disulphide exchange reaction to yield 2-nitromercaptobenzoïc acid (11). This product was measured spectrophotometrically (λ_{max} = 412 nm, ε = 13600 ± 600 M^{-1} cm^{-1}) against an appropriate blank. The oxidized form GSSG was titrated by its reduction by NADPH in the presence of glutathione reductase, the NADPH in excess being measured spectrophotometrically (λ_{max} = 340 nm, ε = 5400 ± 250 M^{-1} cm^{-1}). This titration was carried out with solutions containing an excess of GSSG to avoid any parasitical oxidation of NADPH due to an oxidase activity of glutathione reductase (12).

Hydrogen peroxide concentrations were measured spectrophotometrically by the titanium sulfate method (13, 14) using ϵ_{410} = 700 M^{-1} cm^{-1}.

For the differential spectrophotometric measurements of reaction mixtures, we used two chamber cells, each having an optical path of 0.4375 cm. The reference cell in one chamber contained H_2O_2, (with $[H_2O_2]_0$ = 2 $[H_2O_2]_0$ reaction mixture) and in the other GSH (with $[GSH]_0$ = 2 $[GSH]_0$ reaction mixture). The measure cell in each chamber contained the same reaction mixture.

Another method for determining GSSG was deduced from the differential optical densities at 250 nm. At this wavelength the extinction coefficient for GSH is equal to zero and for H_2O_2 ϵ_{250} = 25 M^{-1} cm^{-1} (15). According to Beer's law, the differential extinction coefficient of GSSG 330 ± 4 M^{-1} cm^{-1} has been determined.

Oxygen concentration was determined by Gilson oxygraph model IC TS-KI. All solutions were prepared just prior to use and buffered at pH 7 in a 10^{-1} *M* phosphate medium. Temperature was maintained constant at 22°C with a BIOBLOCK Polystat circulating water bath.

HPLC analysis has been done according to Reed (16) on a ZORBAX-NH_2 (25 cm × 4.6 mm) column.

Results

1. Differential absorption spectra of the reaction mixture in function of time

For various times after the mixing the differential absorption spectra of a solution containing initially H_2O_2 10^{-3} *M* and GSH 10^{-3} *M* (phosphate buffer 0.1 *M*, pH 7) can be seen in Fig. 1*a* (between 240 and 340 nm). The differential absorption spectrum (*1*) recorded 1 h after the mixing presents two maxima at 250 nm and 305 nm. The absorption band at 250 nm increases (spectra (*2*) and (*3*)), until the end of the reaction (spectrum (4), ΔOD^{250} (5 h 35 min) = 0.16). Simultaneously the absorption band at 305 nm increases and reaches a maximum value at 3 h 20 min (spectrum (*2*), ΔOD^{305} (3 h 20 min) = 0.12) and decays. We note the presence of an isobestic point at 268 ± 8 nm.

The kinetics at 250, 268, and 305 nm are reported on the Fig. 1*b*. The curve (*i*) at 250 nm appears to be composed of a fast increase until ≃ 3 h, followed by a much slower growth until the end of the reaction. This absorption band at 250 nm has been attributed to GSSG ($\Delta\epsilon^{250}$ = 330 M^{-1} cm^{-1}, see Experimental section). At the end of the reaction, the disulfide concentration is equal to 4.85 × 10^{-4} *M* which corresponds to the transfor-

TABLE 1. Determination of the equilibrium constant of formation of the complex [GSH—H_2O_2]

$[H_2O_2]_0$ M	$[GSH]_0$ M	$R = \frac{[GSH]_0}{[H_2O_2]_0}$	$[H_2O_2]_{eq}$ M	$[GSH]_{eq}$ M	$[GSH—H_2O_2]_{eq}$ M	K_2
5×10^{-4}	10^{-3}	2	2.2×10^{-4}	7.2×10^{-4}	2.8×10^{-4}	1770
5×10^{-4}	5×10^{-4}	1	3×10^{-4}	3×10^{-4}	2×10^{-4}	2220
10^{-3}	10^{-3}	1	5×10^{-4}	6×10^{-4}	5×10^{-4}	2000
10^{-3}	8×10^{-4}	0.8	6×10^{-4}	4×10^{-4}	4×10^{-4}	1670
5×10^{-4}	4×10^{-4}	0.8	3.4×10^{-4}	2.4×10^{-4}	1.6×10^{-4}	1960
1.33×10^{-3}	8×10^{-4}	0.6	8.5×10^{-4}	3.2×10^{-4}	4.8×10^{-4}	1760
10^{-3}	6×10^{-4}	0.6	6.5×10^{-4}	2.5×10^{-4}	3.5×10^{-4}	2150
2.5×10^{-3}	10^{-3}	0.4	1.78×10^{-3}	2.2×10^{-4}	7.8×10^{-4}	1990
2.5×10^{-3}	5×10^{-4}	0.2	2.1×10^{-3}	10^{-4}	3×10^{-4}	1900

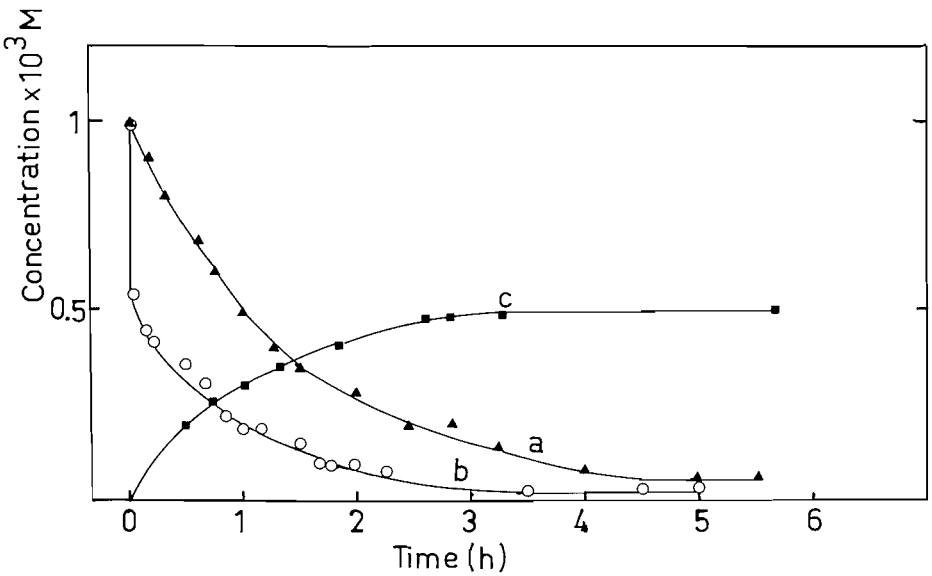


FIG. 2. Evolution of the concentration of GSH (▲), H_2O_2 (○), and GSSG (■). $[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ M, phosphate buffer 0.1 M, pH 7, 22°C.

mation of 97% of GSH initially present in the solution. Consequently, we can consider that the thiol GSH is stoichiometrically oxidized to GSSG within experimental errors. Curve (*iii*) characterizes a transient which exhibits an absorption maximum (at 3 h 20 min), then decays until 7 h, and reaches a final value of ΔOD^{305} (7 h) = 0.035. We will call this intermediate X^{305}. Curve (*ii*) gives the ΔOD^{268} evolution at the isobestic point and it can be observed that a plateau value of $\Delta OD^{268} = 0.095$ is reached and is maintained constant between 3 h 20 min and the end of the reaction. This isobestic point shows that the decay of the intermediate X^{305} gives rise only to GSSG. It can be noticed that ϵ_{305} GSSG = 0 so the absorption at 305 nm is only due to X^{305}. The variations of GSSG concentration during the decay of X^{305} can be measured: it is equal to 6.5×10^{-5} M which represents 13% of the total amount of GSSG appeared. Hence the thiol oxidation via intermediate X^{305} is a minor oxidation phenomenon.

2. *Evolution of GSH, H_2O_2, and GSSG*

Figure 2 shows the kinetic changes in the concentration of GSH, H_2O_2, and GSSG (curves *a*, *b*, and *c*), when the solutions contain initially the same concentrations of GSH and H_2O_2, 10^{-3} M (phosphate buffer 10^{-1} M, pH 7). We remark that the decay of H_2O_2 measured by the titanium composed of two distinct phenomena: the first, a very fast decrease which ends in 2 min and corresponds to the disappearance of 50% of the H_2O_2 initial concentration. The second phenomenon lasts about 6 h, and corresponds to the disappearance of the last 50% of H_2O_2. Identical processes have been observed for numerous experiments; the corresponding initial concentrations are reported in Table 1 (see the three first columns).

The decay of GSH (Fig. 2*a*) and the formation of GSSG (Fig. 2*c*) are contemporary with the slow decrease of H_2O_2 (Fig. 2*b*). We note that, at the end of the reaction, the thiol GSH is quantitatively oxidized to GSSG. This fact confirms the previously stoichiometric oxidation demonstrated according to the results of Fig. 1*b*.

Furthermore, at the end of the reaction the whole H_2O_2 has disappeared (detection by the titanium method), leading us to conclude that 2 mol of H_2O_2 react with 2 mol of GSH to give 1 mol of GSSG. This overall reaction is very different from the usually accepted stoichiometry [1].

[1] $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$

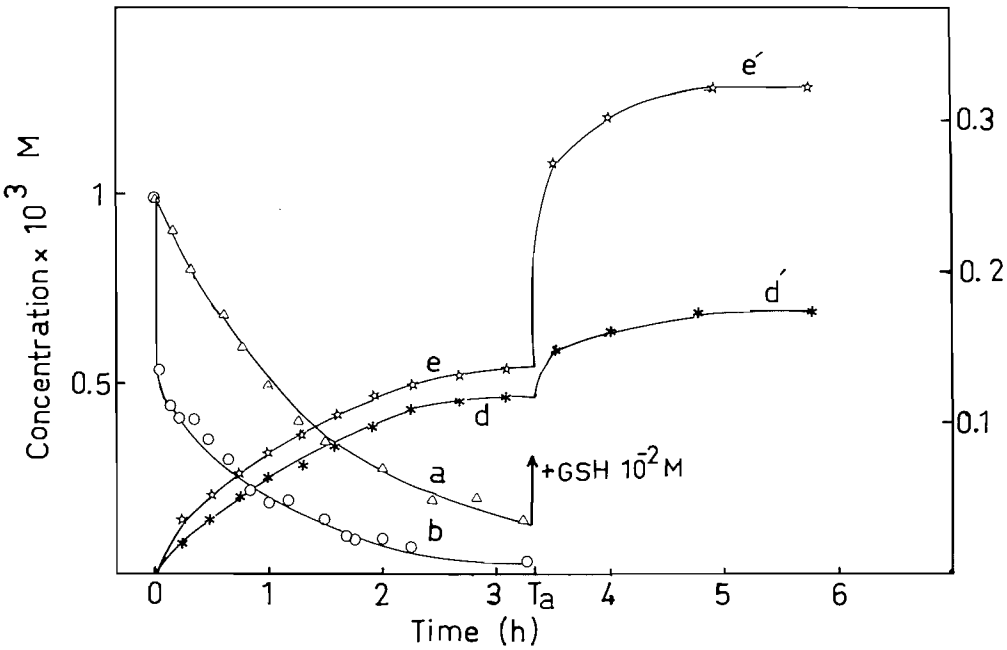


FIG. 3. Addition of GSH at the end of the reaction. Initial reaction: $[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ *M*, phosphate buffer 0.1 *M*, pH 7, 22°C. $[GSH]_t$ (*a*), $[H_2O_2]_t$ (*b*), ΔOD^{250} (*e*), ΔOD^{305} (*d*). T_a = 3 h 20 min, addition of GSH 10^{-2} *M*, ΔOD^{250} (*e'*), ΔOD^{305} (*d'*).

It seems that 1 mol of H_2O_2 disappears without oxidizing the thiol function. To find out what has become with H_2O_2, three experiments have been done.

(a) Oxygen measurements

We have tested the possibility of formation of oxygen by Gilson Oxygraph (see Experimental section) in a solution initially containing GSH and H_2O_2 at the same concentration 10^{-3} *M* (phosphate buffer, pH 7). However, neither at the beginning nor during the reaction was any formation of oxygen observed. Hence H_2O_2 is not oxidized nor does it disproportionate.

(b) Addition of an excess of GSH

An excess of GSH was added (see Fig. 3, time T_a) ($[GSH] \simeq 10^{-2}$ *M*) when H_2O_2 had totally disappeared, i.e. 3 h 20 min after the beginning of the reaction ($[H_2O_2]_0 = [GSH]_0 = 10^{-3}$ *M*, phosphate buffer 10^{-1} *M*, pH 7). We observed a new formation of GSSG (curve *e'*) whose final concentration was equal to 9.85×10^{-4} *M*. Hence this total concentration of GSSG is about the initial H_2O_2 amount (85%), leading us to conclude that an excess of GSH is necessary to reduce quantitatively the whole amount of hydrogen peroxide.

(c) Addition of catalase

To determine the nature of the oxidant property in the final solution, we added catalase 10^{-7} *M*, six hours after mixing the reactants GSH and H_2O_2 ($[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ *M*, phosphate buffer 10^{-1} *M*, pH 7). We observed the formation of oxygen in concentration 5×10^{-4} *M* corresponding to the bound H_2O_2 whereas there was no free H_2O_2 (measured by the titanium method) in the reaction medium.

3. Studies of the X^{305} intermediate

We have shown that the decrease of X^{305} intermediate gives rise to 15% of the total GSSG formed (see Fig. 1*b*). Thus the oxidation process occurring via X^{305} was a minor one. Hence X^{305} cannot be responsible of the oxidant property of the final solution. Nevertheless, to determine the nature of X^{305}, we attempted HPLC analysis of the reaction medium to detect a possible sulfonate product, GSO_3H. We found no GSO_3H because either it was not present or its concentration was too small to be detected by our experimental conditions ($<10^{-5}$ *M*). To determine the chemical behavior of X^{305} we checked the effect of GSH or H_2O_2 on the reaction. An excess of GSH ($\simeq 10^{-2}$ *M*) was added when the differential optical density at 305 nm was maximum (T_a), ($[H_2O_2]_0 = [GSH]_0 = 10^{-3}$ *M*, phosphate buffer 10^{-1} *M*, pH 7). As it can be seen in Fig. 3, we observed that ΔOD^{305} (curve *d'*) increases and remains constant until the end of the reaction. Consequently an excess of GSH protects the X^{305} intermediate and it seems that there is an equilibrium between GSH and X^{305}.

A similar phenomenon has been observed (Fig. 4) when the GSH initial concentration is greater than those of H_2O_2 ($[GSH]_0 = 10^{-3}$ *M*, $[H_2O_2]_0 = 5 \times 10^{-4}$ *M* phosphate buffer 10^{-1} *M*, pH 7). Thus at the end of this reaction ($\simeq$4 h 30 min), free H_2O_2 has totally disappeared (curve *b*), GSSG 5×10^{-4} *M* is formed (curve *e*, ΔOD^{250}), half of GSH is still present (curve *a*) and the X^{305} intermediate reached a plateau value $\Delta OD^{305} = 0.075$ (curve *d*) and does not decrease. In inset of Fig. 4, the differential absorption spectra recorded for different times after mixing are reported. There is no isobestic point because the X^{305} intermediate does not disappear. Therefore we confirm that an initial excess of GSH protects the X^{305} intermediate.

Hydrogen peroxide 2×10^{-3} *M* was added when ΔOD^{305} was maximum (T_b), ($[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ *M*, phosphate buffer 10^{-1} *M*, pH 7). As it can be seen in Fig. 5, the added H_2O_2 accelerates the decrease of X^{305} (curve *d*) giving GSSG (curve *e*). Figure 5 inset shows the differential absorption spectra recorded after the addition of H_2O_2 present an isobestic point at 268 nm, showing that the X^{305} intermediate is transformed into GSSG. We can therefore conclude that H_2O_2 reacts with the X^{305} intermediate to give GSSG.

A similar phenomenon occurs when the initial concentration of H_2O_2 is greater than those of GSH as it can be seen in Fig. 6 ($[GSH])_0 = 10^{-3}$ *M*, $[H_2O_2]_0 = 2.5 \times 10^{-3}$ *M*, phosphate buffer 10^{-1} *M*, pH 7). When ΔOD^{305} is maximum (curve *d*, $t \simeq$

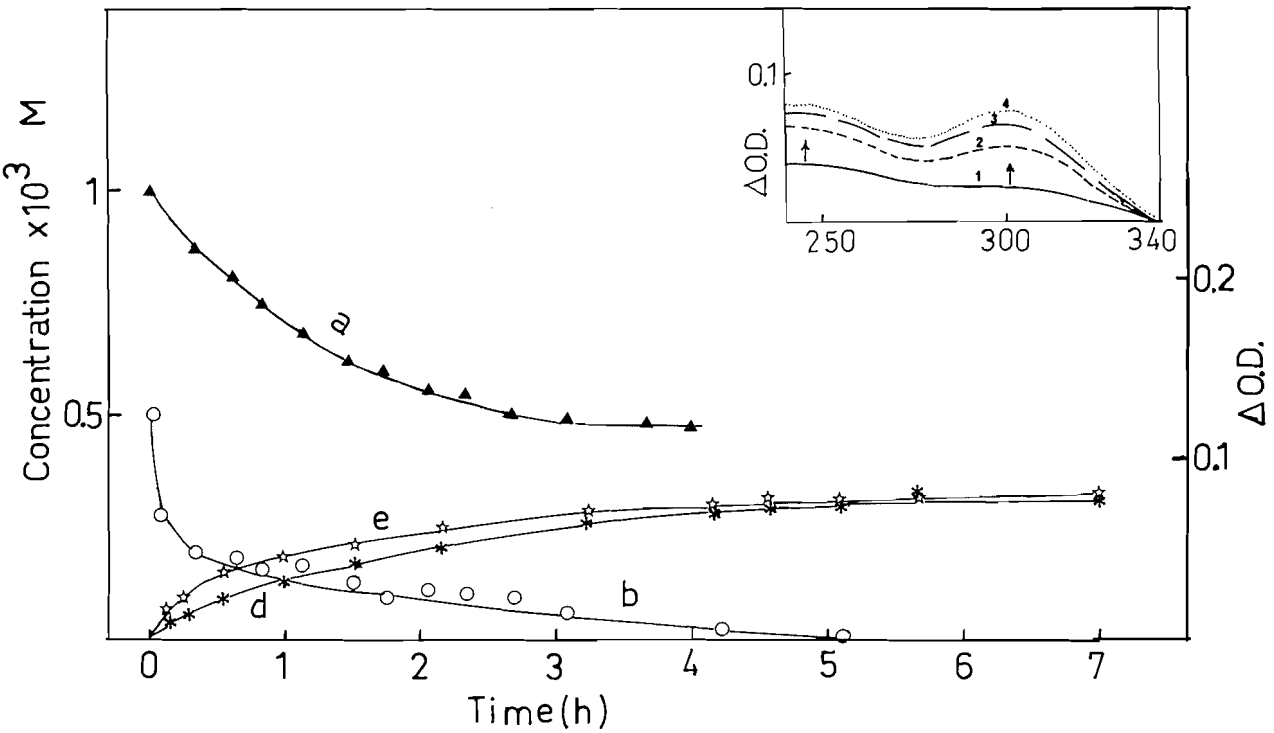


FIG. 4. Kinetic curves of [GSH] (*a*), $[H_2O_2]_0$ (*b*), ΔOD^{250} (*e*), and ΔOD^{305} (*d*). $[GSH]_0 = 10^{-3}$ *M*, $[H_2O_2]_0 = 5 \times 10^{-4}$ *M*, phosphate buffer 0.1 *M*, pH 7, 22°C. Inset: differential absorption spectra of the reaction mixture: (*1*) 32 min, (*2*) 2 h 10 min, (*3*) 3 h 15 min, (*4*) 5 h 7 min after the start of the reaction.

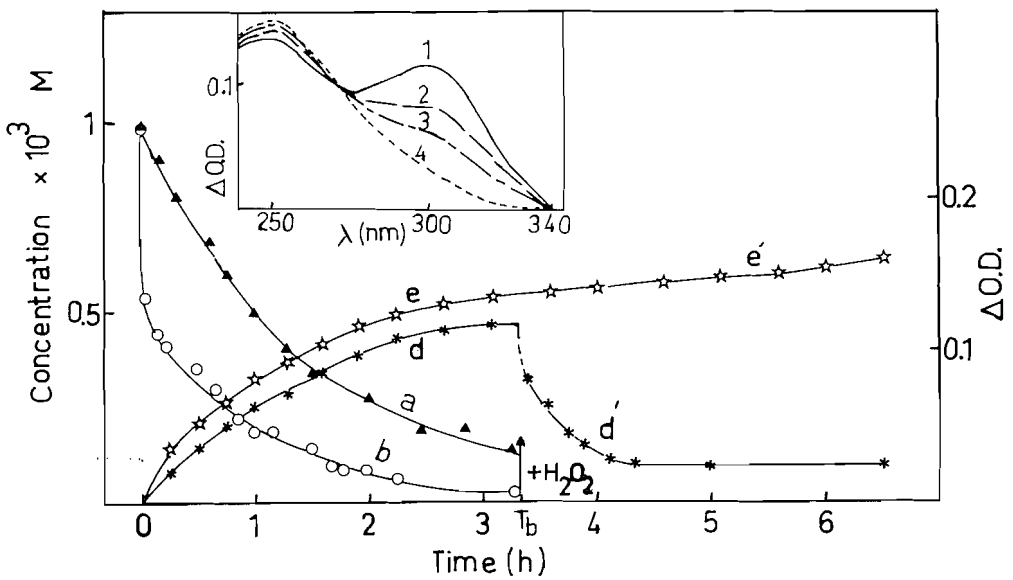


FIG. 5. Addition of $[H_2O_2]$ at the end of a reaction. Initial reaction: $[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ *M*, phosphate buffer 0.1 *M*, pH 7, 22°C. $[GSH]_t$ (*a*), $[H_2O_2]_t$ (*b*), ΔOD^{250} (*e*), ΔOD^{305} (*d*). T_b = 3 h 20 min, addition of H_2O_2 2×10^{-3} *M*, ΔOD^{250} (*e'*), ΔOD^{305} (*d'*). Inset: the differential absorption spectra after the addition of H_2O_2 2×10^{-3} *M*: (*1*) 3 h 20 min (time 0 for the addition of H_2O_2), (*2*) 3 h 25 min, (*3*) 3 h 45 min, (*4*) 4 h after the beginning of the reaction.

50 mn), free H_2O_2 has a high concentration (1.5×10^{-3} *M*, curve *b*) resulting in the X^{305} intermediate decreasing faster than when there is no free H_2O_2 in the medium (Fig. 1*b*, curve *4*). During the decrease of X^{305}, however, hydrogen peroxide remains constant. The decay of X^{305} is thus catalyzed by H_2O_2. Figure 6 inset shows the differential absorption spectra, recorded at different times after the mixing, present an isobestic point at 268 nm when ΔOD^{305} decreases showing the transformation of X^{305} into GSSG.

Discussion

Fast initial decay of H_2O_2

The fast decay of H_2O_2 can be explained by the formation of a compound between GSH and H_2O_2 which could be, for

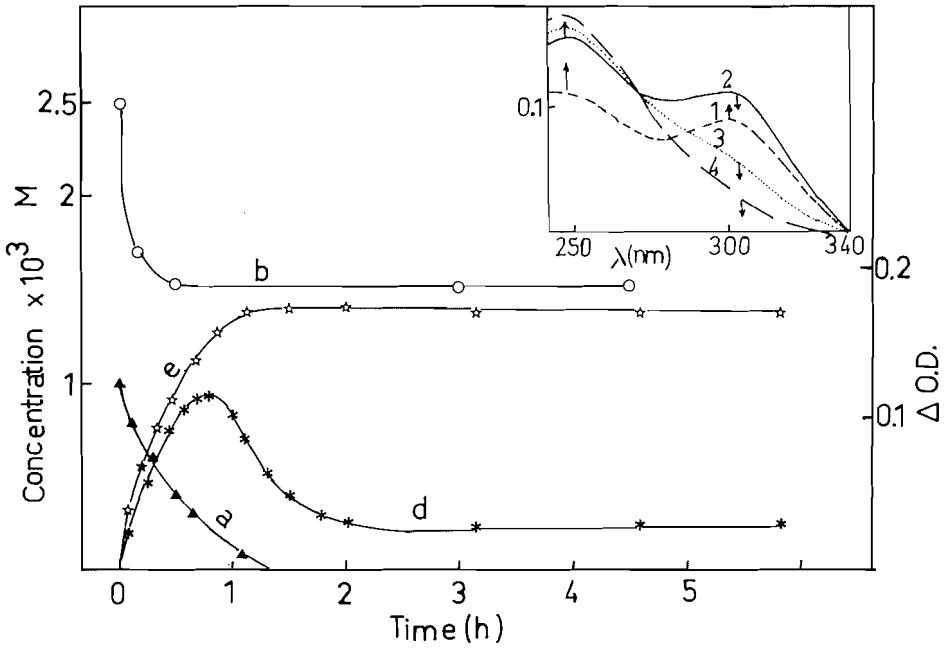


FIG. 6. Kinetic curves of $[GSH]_t$ (*a*), $[H_2O_2]_t$ (*b*), ΔOD^{250} (*e*), and ΔOD^{305} (*d*). $[GSH]_0 = 10^{-3}$ *M*, $[H_2O_2]_0 = 2.5 \times 10^{-3}$ *M*, phosphate buffer 0.1 *M*, pH 7, 22°C. Inset: differential absorption spectra of the reaction mixture: (*1*) 27 min, (*2*) 52 min, (*3*) 1 h 18 min, (*4*) 1 h 45 min after the start of the reaction.

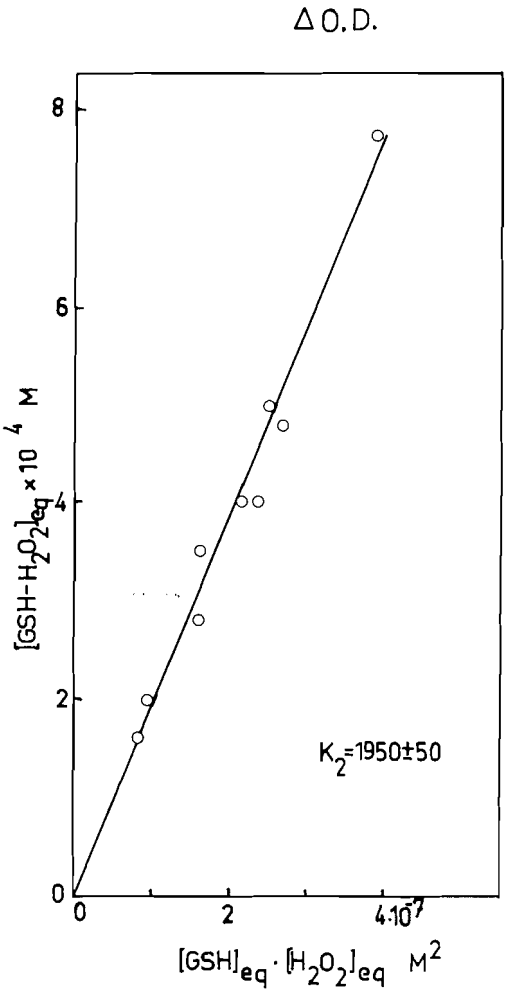
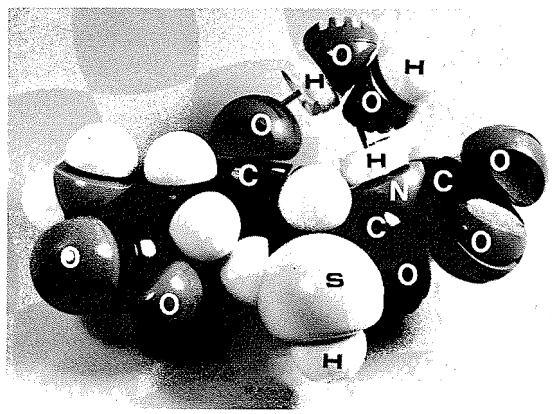


FIG. 7. Determination of the equilibrium constant of formation of the complex [GSH—H_2O_2]. $[GSH—H_2O_2]_{eq}$ vs. $[GSH]_{eq} \times [H_2O_2]_{eq}$, phosphate buffer 0.1 *M*, pH 7, 22°C, $4 \times 10^{-4} \leq [GSH]_0 \leq 2.5 \times 10^{-3}$ *M*, $5 \times 10^{-4} \leq [H_2O_2]_0 \leq 2.5 \times 10^{-3}$ *M*.



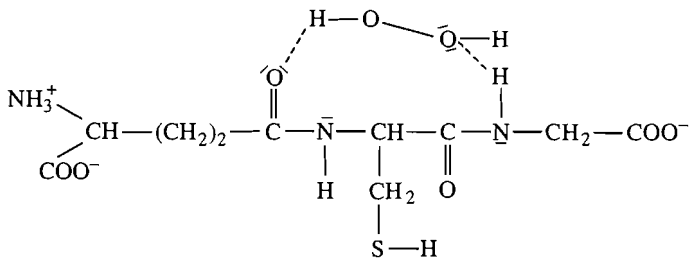


FIG. 8. A chelate model between GSH and H_2O_2.

example, either a peroxide (covalently bond) or a chelate (hydrogen bonds). If we suppose that this product is formed with 1 mol of GSH and 1 mol of H_2O_2, we will use the symbol [GSH—H_2O_2], according to the equilibrium [2]

[2] $GSH + H_2O_2 \rightleftharpoons [GSH—H_2O_2]$ K_2

Since the thiol function of this complex [GSH—H_2O_2] is measured by the Ellman's method (see Experimental section) the curves showing [GSH] versus time would represent the sum [GSH] + [GSH—H_2O_2] versus time. We have to notice that the peroxide or the chelate ([GSH—H_2O_2]) cannot be determined

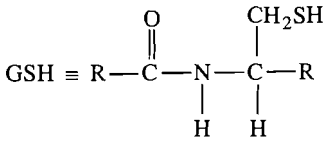
by the titanium method. Let us suppose the concentration of the complex is equal to the concentration of initial disappearance of free H_2O_2. The equilibrium constant is given by

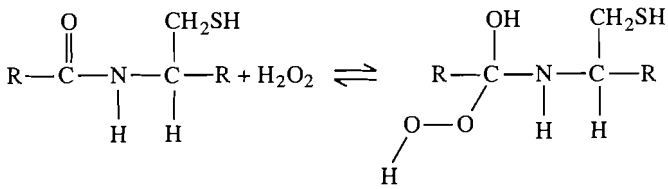
$$K_2 = \frac{[GSH—H_2O_2]_{eq}}{[GSH]_{eq}[H_2O_2]_{eq}}$$

$$= \frac{[H_2O_2]_0 - [H_2O_2]_{eq}}{([GSH]_0 - [H_2O_2]_0 + [H_2O_2]_{eq})[H_2O_2]_{eq}}$$

These values are reported in Table 1. A plot of $[GSH—H_2O_2]_{eq}$ against $[H_2O_2]_{eq} \times [GSH]_{eq}$ is given in Fig. 7, the straight line being consistent with the above mentioned hypothesis. The slope of this line gives $K_2 = 1950 \pm 50$.

Several hypotheses can be considered on the nature of the complex symbolized by $[GSH—H_2O_2]$. It would be a chelate formed from two hydrogen bonds between H_2O_2 and the peptide parts of the glutathione molecule (see Fig. 8 as a model). Another possibility could be a peroxide formed by the nucleophilic addition of H_2O_2 on one of the carbonyl groups of GSH according to





Such an organic peroxide could account for the residual oxidizing power noted experimentally. This type of addition is well known for aldehydes and ketones and, for example, the equilibrium constant between HCHO and H_2O_2 has been determined to be 7000 M^{-1} (17). Thus our value for $K_2 = 1950 \pm 50$ is consistent with this type of addition or adduct formation.

Kinetic scheme

Our experimental results can be explained by two separate kinetic pathways. Pathway I involves reaction [2] and [3]; and pathway II is composed of reactions [4] to [7]

Pathway I

[2] $GSH + H_2O_2 \rightleftharpoons [GSH—H_2O_2]$

[3] $2[GSH—H_2O_2] \rightarrow [GSSG—H_2O_2] + H_2O$

Pathway II

[4] $[GSH—H_2O_2] \rightarrow GSOH + H_2O$

[5] $2GSOH \rightleftharpoons GSH + GSO_2H$

[6] $GSO_2H + [GSH—H_2O_2] \rightarrow [GSSG—H_2O_2] + H_2O_2$

[7] $GSOH + H_2O_2 \rightarrow GSO_2H + H_2O$

In pathway I, equilibrium [2] indicates the disappearance of H_2O_2 without oxidizing the thiol, giving the complex $[GSH—H_2O_2]$ which cannot be distinguished from GSH by the Ellman's method. $[GSH—H_2O_2]$ could disproportionate according to reaction [3], giving the final product $[GSSG—H_2O_2]$ which accumulates. The formation of this peroxide or chelate between GSSG and H_2O_2 is consistent with the following experimental results: at the end of a reaction, when there is no free H_2O_2 (*i*) the addition of catalase stimulates the formation of oxygen (see Fig. 2, curve *c*) and (*ii*) the presence of an excess of GSH gives an amount of GSSG equal to those of the hound peroxide (see Fig. 3, curve *e'*).

In pathway II, reaction [4] occurs in competition with reaction [3] and could explain the formation of another oxidized from of GSH like, for example, GSOH, which is named X^{305} in the text. This reaction which can be described as a nucleophilic attack on H_2O_2 initiates the minor oxidation mechanism. It is consistent with previous works where H_2O_2 acts with thiocyanate (6), 2-mercaptoethanol (8), hydrogen sulfide (2), thiourea or *N*,*N'*-dialkylthioureas (4) giving an intermediate type of RSOH.

We have admitted that GSOH diproportionates according to reaction [5] giving GSH and GSO_2H, this reaction being similar to those of CySOH (18) and HSOH (2). The backward reaction [−5] could explain the stabilization of the intermediate GSOH when an excess of GSH is present at the end of a reaction (see Fig. 3). GSO_2H would react with $[GSH—H_2O_2]$ giving $[GSSG—H_2O_2]$ according to reaction [6]. Both reactions [5] plus [6] explain the transformation of GSOH (= X^{305}) into $(GSSG—H_2O_2]$ (λ_{max} = 250 nm) that is consistent with the presence of an isobestic point (268 nm) when the intermediate X^{305} decays (see Figs. 1, 5, and 6, curves *iii*, *d'*, *d*); reaction [7] takes place only when there is an excess of free H_2O_2 in the medium and could explain the fast decrease of X^{305} (see Figs. 5 and 6). The sum of the reactions [7] plus [6] involves the non-decomposition of H_2O_2 as it has been experimentally verified (see Fig. 6, curve *d*), the decay of X^{305} being catalyzed by H_2O_2.

Simulation of the kinetic curves have been done by four successive steps; one step for pathway I and three other steps for pathway II.

(i) Pathway I: We have calculated only with pathway I (reactions [2], [−2], and [3]), the three rate constants k_2, k_{-2}, and k_3 (knowing the experimental constant $K_2 = 1950 \pm 50$) with the following values:

$$k_2 \geq 15\ M^{-1}\ s^{-1}$$

$$k_{-2} \geq 7.5 \times 10^{-3}\ s^{-1}$$

$$K_2 = 2000 \pm 700$$

$$k_3 = 0.9 \pm 0.1\ M^{-1}\ s^{-1}$$

The kinetic scheme [2], [−2], and [3] is consistent with 85% of the overall oxidation of GSH and the calculated curves agree with experimental ones during 1.5 to 2 half times (calculated curves not shown).

Several comments can be made. The quantitative treatment of the kinetic scheme shows that reaction [3] is the rate-limiting step for GSH oxidation. Indeed rate constant k_3 (0.9 $M^{-1}\ s^{-1}$) is smaller than the rate constant of the initiation reaction k_2 (≥ 15 $M^{-1}\ s^{-1}$). We gave the lower limit values of the rate constants k_2 and k_{-2} which are consistent with the k_3 limiting step value (the ratio k_2/k_{-2} being constant). The slow decay of free H_2O_2 can be explained by the displacement of equilibrium [2] to the right by reaction [3]. Two reactions [2] and [3] correspond to the overall reaction:

[8] $2GSH + 2H_2O_2 \rightarrow [GSSH—H_2O_2] + 2H_2O$

We have calculated that 80–85% of the total of $[GSSG—H_2O_2]$ is formed according to both reactions [2] and [3] ($k_2 \geq 15$ $M^{-1}\ s^{-1}$, $k_{-2} \geq 7.5 \times 10^{-3}\ s^{-1}$, $K_2 = 2000 \pm 700$, $k_3 = 0.9 \pm 0.1\ M^{-1}\ s^{-1}$).

(ii) Pathway II: This pathway involves three other steps. We have determined the rate constant k_4 of reaction [4] which initiates the minor oxidation process by studying the kinetic formation of X^{305} We have made the following hypothesis: since the disappearance of X^{305} gives the formation of GSSG,

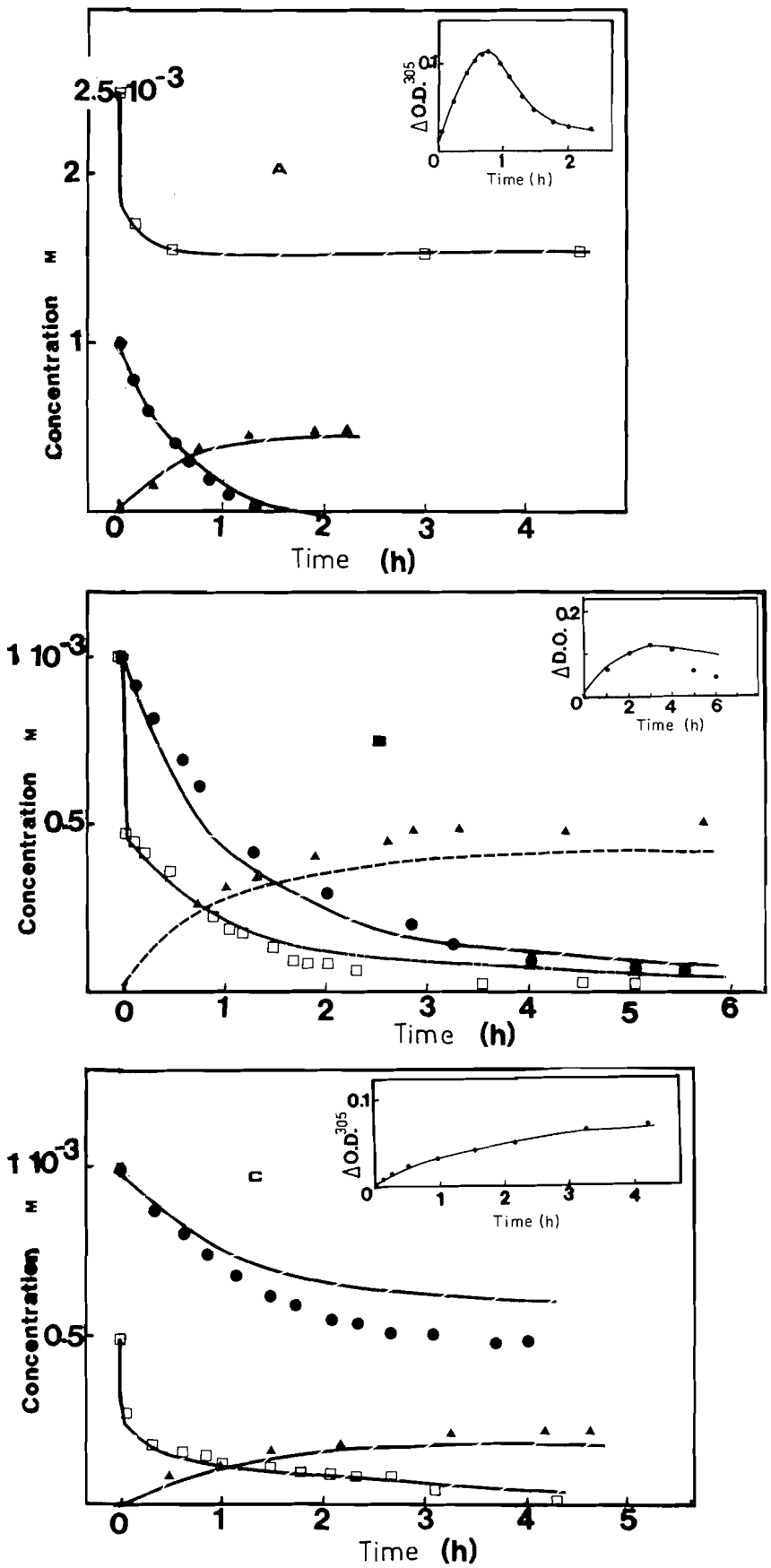


FIG. 9. Comparison between the computed kinetic curves and the experimental points [GSH] + [GSH − H_2O_2] (●), [H_2O_2] (□), [GSSG—H_2O_2] (▲) for three different experiments: (*A*) $[GSH]_0 = 10^{-3}$ *M*, $[H_2O_2]_0 = 2.5 \times 10^{-3}$ *M*; (*B*) $[GSH]_0 = [H_2O_2]_0 = 10^{-3}$ *M*; (*C*) $[GSH]_0 = 10^{-3}$ *M*, $[H_2O_2]_0 = 5 \times 10^{-4}$ *M*, phosphate buffer 0.1 *M*, pH 7, 22°C. Inset: computed curves of ΔOD^{305} (taking $\Delta\epsilon^{305} = 750 \pm 50$ M^{-1} cm^{-1}, see text) for three experiments (points are experimental).

we supposed that the variation $\Delta[X^{305}]$ was equal to [GSSG]/2. Therefore

$$\Delta\epsilon X^{305} = \frac{\Delta OD^{305}}{\Delta[GSSG]/2} = 750 \pm 50\ M^{-1}\,cm^{-1}$$

Using this method we could obtain the evolution of X^{305} concentration and consequently the initial rate of formation of X^{305}. Thus from different experimentations we have obtained the mean value of k_4 which is equal to $(1.5 \pm 0.5) \times 10^{-4}\ s^{-1}$.

The second step was the determination of k_7 and k_6. As we have seen before in the experimental results (paragraph 3), the decay of X^{305} is accelerated by added H_2O_2 (Fig. 5). This phenomenon has also been seen when $[H_2O_2]_0 > [GSH]_0$ (Fig. 6). Hence we have supposed that reactions [7] and [6] were predominant under these conditions and that reactions [5] and [−5] can be ignored.

k values of reactions [7] and [6] have been calculated using the kinetic scheme composed of reactions [2], [−2], [3], [4], [7], and [6]. The following k values gave the best agreement with the experimental curves in Fig. 6:

$$k_7 = 0.25 \pm 0.05\ M^{-1}\,s^{-1}$$

$$k_6 = 30 \pm 5\ M^{-1}\,s^{-1}$$

The third step consists of the calculation of the rate constants k_5 and k_{-5} in the case where $[GSH]_0 \geq [H_2O_2]_0$ (Fig. 9*C*). We then used the total kinetic scheme comprising reactions [2], [−2], [3], [4], [5], [−5], [6], and [7] to obtain

$$k_5 = 0.05 \pm 0.02\ M^{-1}\,s^{-1}$$

$$k_{-5} = 0.06 \pm 0.01\ M^{-1}\,s^{-1}$$

From these we obtain

$$K_5 = 0.83 \pm 0.17$$

Thus this reaction mechanism (reactions [2] to [7]) has been shown to adequately describe all kinetic results (Fig. 9 *A*, *B* and *C*).

Figure 9 *A*, *B*, and *C* insets show the calculated curves of ΔOD^{305}, points are experimental. We can see a good agreement with the experimental values.

When there is initially an excess of GSH it can be supposed that another reaction takes place, for example,

[9] $[GSSG—H_2O_2] + 2GSH \rightarrow 2GSSG + 2H_2O$

This reaction explains the results reported before showing that an excess of GSH gives an amount of GSSG equal to those of the peroxide $[GSSG—H_2O_2]$.

Reactions [8] and [9] add together to give reaction [1], in good agreement with the usually accepted overall reaction:

[1] $2GSH + H_2O_2 \rightarrow GSSG + 2H_2O$

Hence when there is a sufficient excess of GSH, the overall stoichiometry of the reaction could be accurately expressed by reaction [1].

Conclusion

The analytical methods used in this work allowed us to measure (*i*) free H_2O_2 and not other peroxide with the titanium method, (*ii*) the SH group with Ellman's method, (*iii*) the disulfide S—S with the enzymatic NADPH reduction or with the differential absorption at 250 nm, (*iv*) the intermediate X^{305} (which could be GSOH) with the differential absorption at 305 nm.

Our kinetic data indicate that in our experimental conditions $(0.2 \leq [GSH]_0/[H_2O_2]_0 \leq 2)$ the overall oxidation equation has been established to be 2 mol GSH with 2 mol of H_2O_2 giving 1 mol GSSG:

[8] $2GSH + 2H_2O_2 \rightarrow [GSSG—H_2O_2] + 2H_2O$

This implicates that an important part of H_2O_2 disappears without oxidizing the thiol function. We attributed this result to the formation of a peroxide or a chelate between GSH and H_2O_2, in which bound H_2O_2 cannot be measured by our titanium sulphate method.

Since GSH can rapidly scavenge H_2O_2 before reducing it, GSH appears as a carrier of H_2O_2 and therefore as a protector against the toxicity of H_2O_2.

Moreover, it has been shown that the oxidation of GSH by H_2O_2 has also a minor oxidation process (15% of the total GSSG formation). This phenomenon is mediated by an intermediate compound absorbing at 305 nm, which has been seen by McCormick *et al.* (9) in the same conditions.

Acknowledgments

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