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OXIDATION OF FERROCENE AND SOME SUBSTITUTED FERROCENES IN THE PRESENCE OF HORSERADISH PEROXIDASE +

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Summary

The enzyme catalysed oxidation of ferrocene and some substituted ferrocenes to the corresponding ferricinium ions by hydrogen peroxide in the presence of native or immobilized horseradish peroxidase has been studied. Initial and maximum rates of oxidation have been determined. It was found that the oxidation was independent of the hydrogen peroxide concentration. The oxidation of ferrocene was effected also by horseradish peroxidase in a coupled system with glucose oxidase in the absence of any added hydrogen peroxide.

Introduction

Ferrocene is stable in air but in acid solution in the presence of oxygen it readily loses an electron to give the ferricinium ion. Oxidation of ferrocene can be effected with a wide range of organic and inorganic oxidizing agents

+ Some of these results have been presented in a preliminary form [1]

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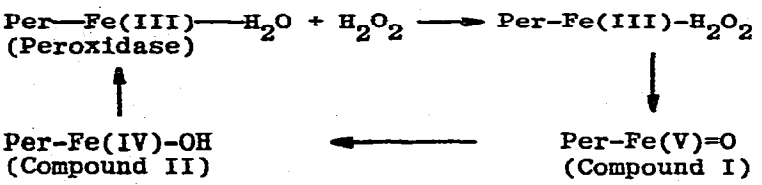
and also electrochemically [2]. A convenient method of oxidation is to treat ferrocene with iron(III) chloride when ferricinium tetrachloroferrate is produced [3]. This oxidation by iron(III) is similar to that found in reactions catalysed by the enzyme peroxidase. In the presence of hydrogen peroxide, peroxidase forms compounds that contain iron(IV) and iron(V) and these species participate in the oxidation process (Scheme 1) [4]



Scheme 1

Horseradish peroxidase (HRP) catalyses the oxidation of some iron(II) complexes to the corresponding iron(III) complexes. For example ferrocytochrome C is oxidized to ferricytochrome C and also hexacyanoferrate(II) ion is oxidized to hexacyanoferrate(III) ion (Scheme 2) [5,6].

$$HRP + H_2O_2 \longrightarrow \text{Compound I.}$$

$$\text{Compound I} + [Fe(CN)_6]^{4-} \longrightarrow \text{Compound II} + [Fe(CN)_6]^{3-}$$

$$\text{Compound II} + [Fe(CN)_6]^{4-} \longrightarrow HRP + [Fe(CN)_6]^{3-}$$

Scheme 2

The direct oxidation of ferrocene by acidic hydrogen peroxide has been shown to be slow and autocatalytic and we decided to investigate the effect of horseradish peroxidase on this system [7]. Very few examples of enzymic reactions involving the ferrocene molecule have been reported. Johnson and Murray hydroxylated α-oxo-1,2-tetramethyleneferrocene with the mould Sporotrichum sulfuresens to give oxo-6--hydroxyferroceno[1,2]cyclohex-1-en-3-one. This was an oxygenation by an enzymic system in the presence of a ferrocene moiety [8].

Recently, ferricinium ion has been employed for the redox titration of cytochrome c and cytochrome c oxidase. In this reaction the ferricinium ion-ferrocene couple was acting as a mediator to couple the electron transfer between the electrode and the heme proteins [9].

In this report, we describe the oxidation of ferrocene and some substituted ferrocenes by hydrogen peroxide in the presence of horseradish peroxidase.

Results and discussion

Hexacyanoferrate(II), which is readily oxidised by hydrogen peroxide in the presence of HRP has a standard reduction potential of -0.36 V which is similar to the value for ferrocene (-0.56 V) [10]. Also the electron-exchange-rate constant for ferrocene is approximately 9 x 10^3 times greater than for hexacyanoferrate(II). These results suggested that ferrocene should be oxidized readily by hydrogen peroxide in the presence of HRP. The addition of HRP and hydrogen peroxide to a yellow solution of ferrocene in aqueous methanol gave a blue solution [7,11] whilst controls in the absence of either HRP or hydrogen peroxide remained yellow for several hours. The addition of ammonium diamminetetrathiocyanatochromate(III) (ammonium Reineckate) to the blue solution gave a precipitate of ferricinium diamminetetrathiocyanatochromate(III) [12]. The corresponding salts of ferrocenemethanol and 6-(2--ferrocenylacetamido)penicillanic acid [13] were isolated and characterised.

The rates of oxidation of ferrocene and some substituted ferrocenes were determined spectrophotometrically, (Table 1). The results indicate that the introduction of a substituent onto the η-cyclopentadienyl ring reduces the rate of oxidation. This presumably is a steric effect with the approach of the enzyme to the substituted ferrocene being restricted. An at-

tempt was made to oxidize ferrocenoic acid and dimethylaminomethylferrocene in the presence of HRP but no oxidation was detected spectrophotometrically. When hydroxyferrocene and methoxyferrocene were treated with hydrogen peroxide in the presence of HRP a brown precipitate of iron(III) oxide was formed from the decomposition of the ferrocene molecules. Thin-layer chromatography of the solution of hydroxyferrocene after treatment with HRP showed that there were no new ferrocene species present and this indicated that only oxidative decomposition had occurred. This was unexpected as phenols undergo a peroxidatic reaction, for example p-cresol gives 2,2'-dihydroxy-5,5'-dimethyldiphenyl, the corresponding triphenyl derivative and a substituted furan [14].

The oxidative decomposition of methoxyferrocene in the presence of HRP and hydrogen peroxide was followed by measuring the rate of appearance of iron(III) (Fig. 1). Control experiments showed that both HRP and hydrogen peroxide were necessary to effect the liberation of iron(III). A similar experiment with ferrocene failed to liberate iron(III); only the ferricinium ion was formed.

The effect of varying the peroxide concentration on the rate of oxidation of ferrocene in the presence of HRP was investigated (Table 2). An approximately seventeen-fold increase in hydrogen peroxide concentration had little effect on the rate. This is characteristic of an enzyme catalysed reaction at saturation kinetics. The radical inhibitor 4-t-butylcatechol was added to the enzyme system [15]. As the concentration of the inhibitor increased the induction period prior to ferricinium ion formation increased (Fig. 2). This suggested that the oxidation in the presence of HRP was a radical reaction propagated by the intervention of another oxidant [5,15].

TABLE 1

Rates of reaction of ferrocene and some ferrocene derivatives with H_2O_2 in the presence of HRP

Substrate	Substrate Concentration μ mol	Initial Rate ΔA/μg enzyme /μ mol substrate[a]		Maximum Rate ΔA/μg enzyme /μ mol substrate[a]	
		Native Enzyme	Bound Enzyme[b]	Native Enzyme	Bound Enzyme[b]
FcH	5.4	0.026	0.0046	0.0099	0.0019
$FcCH_2OH$	7.2	0.012	0.0029	0.0050	0.0013
FcCHMeOH	6.2	0.012	0.0028	0.0056	0.0011
$FcCMe_2OH$	5.6	0.010	0.0028	0.0048	0.0011
$FcCH_2CO_2H$	5.85	0.0016	0.00011	0.00063	0.00006
FcHgCl	3.75	0.0011	0.00021	0.00039	0.00007
$FcB(OH)_2$	6.8	0.0004	0.00002	0.00014	0.00001
$FcCH\overset{+}{N}Me_3\bar{I}$*	15.1	0.0003	0.00005	0.00016	0.00002

* performed in aqueous buffer, with background H_2O_2 oxidation rate of 0.0001 ΔA/min/μmol substrate

[a] ΔA - change in absorbance

[b] 42.9 mg of HRP bound per gram of dry polymer

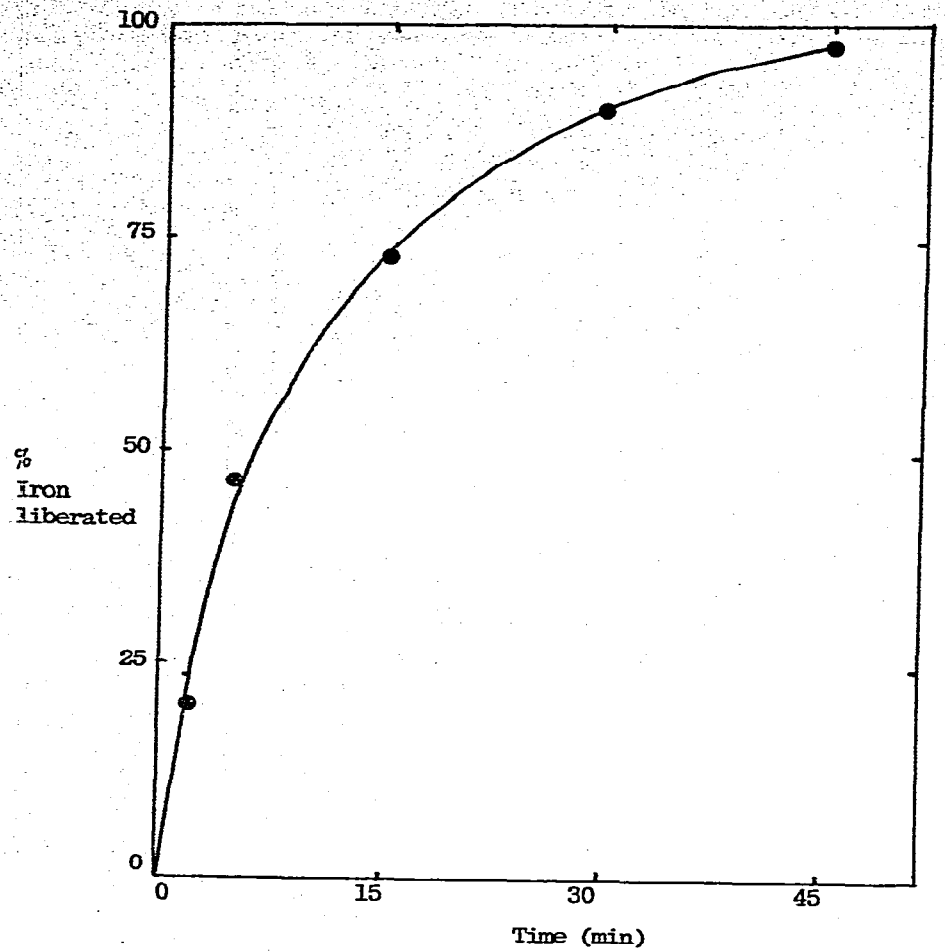


Figure 1 – The liberation of iron(III) from the oxidative decomposition of methoxyferrocene by native HRP and hydrogen peroxide

A potential difficulty in studying enzyme catalysed reactions on hydrophobic molecules such as ferrocene is to secure adequate dissolution of the enzyme molecules in the necessary aqueous/organic medium. To overcome this problem we have devised a method whereby the enzyme is attached to a polymer which is capable of gelation in both water and organic solvents [16]. Covalent coupling of the enzyme with the aqueous-gel network followed by exchange of the solvent component of the gel by the aqueous/organic mixture being

used enables a suitable molecular dispersion of the enzyme to be obtained without risk of precipitation.

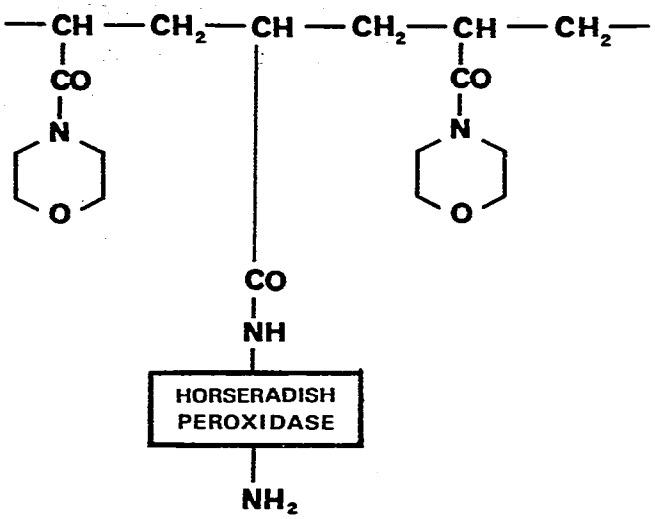


TABLE 2

The effect of change in hydrogen peroxide concentration on the rate of oxidation of ferrocene in the presence of native HRP

Hydrogen peroxide concentration M	Initial Rate $\Delta A\ min^{-1}$*
1.75×10^{-6}	0.0052
3.03×10^{-6}	0.0051
7.06×10^{-6}	0.0056
7.50×10^{-6}	0.0050
1.50×10^{-5}	0.0060
3.00×10^{-5}	0.0055

* Ferrocene and enzyme concentrations were kept constant as the concentration of hydrogen peroxide was changed.

An immobilized form of HRP was prepared by covalent binding of the enzyme to a poly(acryloylmorpholine) xerogel network in the bead form [16]. The xerogel network was

prepared by aqueous suspension co-polymerization of acryloylmorpholine and N-acryloyl-N'-t-butoxycarbonylhydrazine (molar ratio 10:1). The xerogel was treated with acid to remove the N-t-butoxycarbonyl groups, sodium nitrite added to generate the corresponding azide and this was coupled with HRP to give the immobilized enzyme system (I). A suspension of the immobilized enzyme was stirred with ferrocene in the presence of hydrogen peroxide, the solution was centrifuged and the supernatant was monitored spectrophotometrically. Oxidation of ferrocene to the ferricinium ion readily occurred and the initial and maximum rates of reaction for a series of substituted ferrocenes were determined (Table 1). The initial rate measurements show that oxidation was slower, in the presence of the immobilized enzyme. These results demonstrate that the poly(acryloylmorpholine)-gel network is a useful immobilization matrix for the study of enzyme reactions in aqueous/organic solvent mixtures and this is in agreement with our earlier work on carbonic anhydrase [16].

Peroxidase catalyses the oxidation of a substrate by hydrogen peroxide. When HRP is used in conjunction with glucose oxidase it is not necessary to add hydrogen peroxide to effect an oxidation. The glucose oxidase utilizes molecular oxygen in the presence of β-D-glucose to give D-glucono-S-lactone and hydrogen peroxide and the lactone is hydrolysed to gluconic acid (Equations 1 and 2) [17].

$$\beta\text{-D-glucose} + O_2 \xrightleftharpoons{\text{glucose oxidase}} \text{D-glucono-S-lactone} + H_2O_2 \quad (1)$$

$$\text{D-glucono-S-lactone} + H_2O \rightleftharpoons \text{gluconic acid} \quad (2)$$

The hydrogen peroxide generated can then be utilized by peroxidase in a further oxidation step. Glucose oxidase coupled with HRP was added to a solution of ferrocene or ferrocenylpropan-2-ol in 50% methanol – acetate buffer in the

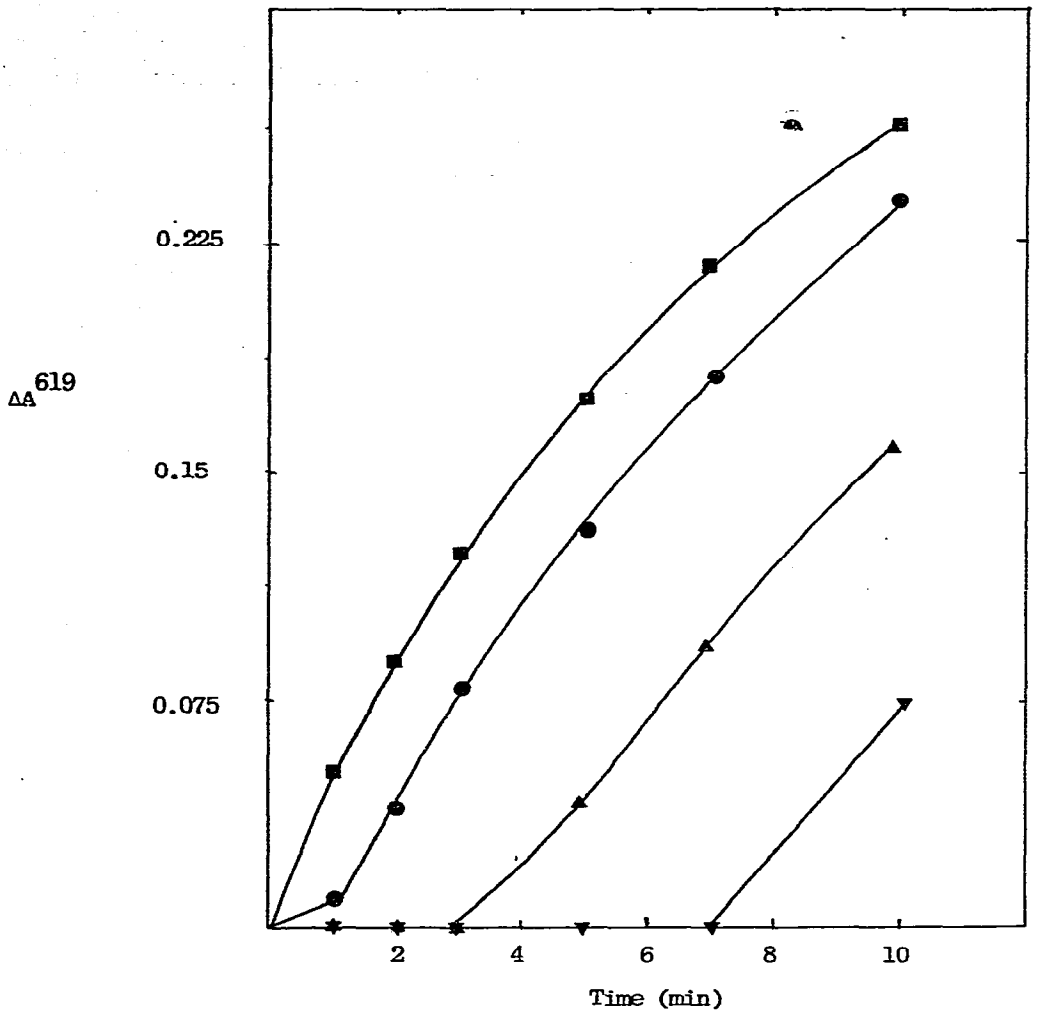


Figure 2 - The effect of 4-t-butylcatechol on the rate of the native peroxidase catalysed oxidation of ferrocene (■) 0; (●) 0.52; (▲) 1.04 and (▼) 1.56 μmoles of 4-t-butylcatechol

presence of β-D-glucose. Oxidation of ferrocene and ferrocenylpropan-2-ol to the corresponding ferricinium ions occurred and the reactions were monitored spectrophotometrically. The oxidations proceeded relatively slowly but they demonstrated that the reaction was enzyme catalysed and it was unnecessary to add hydrogen peroxide to effect oxidation.

Experimental

Oxidation of ferrocene with hydrogen peroxide in the presence of native HRP

Ferrocene (0.0084 g) was dissolved in methanol (25 cm^3) and to an aliquot (3 cm^3) of this solution was added 0.1 M potassium phthalate buffer (pH 6.1, 2.3 cm^3) and 0.005 mM hydrogen peroxide (0.2 cm^3). Horseradish peroxidase (E.C. 1.11.1.7) (Sigma Type II, 0.6% in water) (0.5 cm^3) was added and the rate of oxidation was followed by the change in absorbance at 619 nm [11, 18] at 25 °.

The same reaction was carried out in the absence of HRP and the change in absorbance at 619 nm was negligible over 4 h.

Oxidation of ferrocene in the presence of HRP at different hydrogen peroxide concentrations

The oxidation of ferrocene was carried out as previously described except that the concentration of hydrogen peroxide was varied (Table 2).

Effect of 4-t-butylcatechol on the oxidation of ferrocene by hydrogen peroxide in the presence of HRP

The oxidation of ferrocene was carried out as previously described except that 4-t-butylcatechol (Fig. 2) was dissolved in the buffer solution before it was added to the solution of ferrocene in methanol.

Oxidation of substituted ferrocenes

The oxidations with native HRP of some substituted ferrocenes (Table 1) were carried out as described for ferrocene. The solutions were monitored between 610 - 640 nm. The wavelength was determined, prior to the rate determination, from solutions of the substituted ferrocene and the corresponding ferricinium ion.

Oxidation of ferrocene with hydrogen peroxide in the presence of immobilized HRP

The HRP was immobilized on a poly(acryloylmorpholine)/ N-acryloyl-N'-t-butoxycarbonylhydrazine copolymer following standard procedures.[16]. The immobilized HRP [0.0015 g in phthalate buffer (0.5 cm^3)], 0.1 M phthalate buffer (2.3 cm^3) and 0.005 mM hydrogen peroxide (0.2 cm^3) were added to the solution of ferrocene in methanol. The solution was stirred and prior to the absorbance reading, it was centrifuged and an aliquot of the supernatant removed. The absorbance was recorded and the solution was returned immediately to the centrifuged polymer and the reaction was allowed to proceed.

This procedure was repeated with a series of substituted ferrocenes (Table 1).

Ferricinium diamminetetrathiocyanatochromate(III)

Ferrocene (0.0084 g) was dissolved in methanol (25 cm^3) and 0.1 M phthalate buffer (19.2 cm^3) and 0.005 mM hydrogen peroxide (1.7 cm^3) were added. HRP (4.2 cm^3) was added and the oxidation was allowed to proceed for 4 h. A saturated solution of ammonium diamminetetrathiocynatochromate(III) (50 cm^3) was added and ferricinium diamminetetrathiocyanatochromate(III) (0.014 g, 59%) was precipitated. The precipitate was collected by centrifugation, washed with water and dried. The ferricinium salt was identical (infrared) with an authentic sample [12] (Found: C, 32.96; H, 3.54; Cr, 9.76; $C_{14}H_{16}CrFeN_6S_4$ requires: C, 33.33; H, 3.19; Cr, 10.31%).

Ferricinium salts of some substituted ferrocenes

The diamminetetrathiocyanatochromate(III) salts of ferrocenemethanol (0.0108 g) and 6-(2-ferrocenylacetamido)penicillanic acid sodium salt were prepared as described above.

Hydroxymethylferricinium diamminetetrathiocyanatochromate(III) (0.012 g, 44%) mp. >320 ^{o} (Decompn.)

(Found: C, 33.80; H, 3.61; Cr, 9.55; $C_{15}H_{18}CrFeS_4N_6O$ calcd.: C, 33.71; H, 3.39; Cr, 9.73%).

6-(2-ferriciniumacetamido)penicillanic acid sodium salt diamminetetrathiocyanatochromate(III) m.p. >320 °C (Decompn.) (Found: C, 37.73; H, 3.96; Cr, 5.71; $C_{24}H_{27}CrFeN_8NaO_4S_5$ calcd.: C, 38.83; H, 3.48; Cr, 6.64%).

Oxidation of ferrocene and ferrocenylpropan-2-ol with glucose oxidase and HRP

Ferrocene (0.0018 g) was dissolved in methanol (3 cm^3) and 0.1 M sodium acetate buffer (pH 6.4) (1 cm^3), 1% glucose solution (1 cm^3) and HRP (Sigma Type II, 3% in acetate buffer) (0.5 cm^3) were added. Glucose oxidase (E.C. 1.1.3.4) (Sigma Type II, 7% in acetate buffer) (0.5 cm^3) was added. The rate of oxidation was followed by the change in absorbance at 619 nm.

Ferrocenylpropan-2-ol (0.0021 g) in methanol (3 cm^3) was oxidized with a glucose oxidase - HRP mixture as described for ferrocene. The solution was monitored spectrophotometrically at 630 nm. (For ferrocene the initial and maximum rates were 0.21 and 0.12 $\Delta A/h/\mu mol$ substrate and the corresponding values for ferrocenylpropan-2-ol were 0.12 and 0.08 $\Delta A/h/\mu mol$ substrate.

Oxidative decomposition of methoxyferrocene by hydrogen peroxide in the presence of HRP

Methoxyferrocene (0.0117 g) was dissolved in methanol (25 cm^3) and to a series of aliquots (3 cm^3) of this solution were added 0.1 M potassium phthalate buffer (2.3 cm^3), 0.005 mM hydrogen peroxide (0.2 cm^3) and HRP (Sigma Type II, 1.1% in water) (0.5 cm^3). The time was recorded when the enzyme was added and after different time intervals (Fig. 1) the reaction was stopped by the addition of acid followed by ether extraction to remove unreacted ferrocene. 0.1 M

Ammonium thiocyanate (0.3 cm^3) was added to the aqueous layer (3.0 cm^3) and the absorbance was recorded immediately at 480 nm [19]. The iron(III) concentration was calculated from a calibration curve.

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