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# PEROXIDASE CATALYSED OXYGENATION OF 4,2′,4′-TRIHYDROXYCHALCONE

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**Key Word Index**—*Cicer arietinum*; Leguminosae; peroxidase; oxygenation; 4,2′,4′-trihydroxychalcone; 1,2-dioxetane; 7,4′-dihydroxydihydroflavonol; 6,4′-dihydroxy-2-(α-hydroxybenzyl)coumaranone; 6,4′-dihydroxyaurone; 7,4′-dihydroxyflavonol.

**Abstract**—Biochemical studies of the peroxidase-catalysed aerobic oxidation of 4,2′,4′-trihydroxychalcone to yield the cyclic peroxide (1,2-dioxetane) of the chalcone are reported. Comparative studies were made with partially purified enzyme from garbanzo seedlings and with commercial horseradish peroxidase (HRP). Oxygen is obligatory in the reaction and is consumed in equimolar amount with chalcone. Hydrogen peroxide is also required, but only a catalytic net consumption occurs. Redogenic donors strongly inhibit chalcone oxidation as do $Mn^{2+}$, mercaptoethanol and diethyldithiocarbamate. 7,4′-Dihydroxyhydroflavonol and 6,4′-dihydroxy-2-(α-hydroxybenzyl)coumaranone are also formed as minor products of oxidation of the chalcone by purified peroxidase. The effects of various additives and hydrogen donors on the relative proportions of the different oxidation products have been studied and likely biochemical and chemical mechanisms of formation of these compounds are presented. A non-enzymic radical chain mechanism for chalcone peroxide formation accounts for the apparent oxygenase activity of peroxidase in these reactions.

## INTRODUCTION

In the previous paper [1], the isolation and characterisation of the chalcone cyclic peroxide (**2**) and the benzoxepinone-spiro-cyclohexadienone derivative OC (**3**) as major products of the peroxidase-catalysed oxidation of 4,2′,4′-trihydroxychalcone (**1**) was reported. The cyclic peroxide (**2**) was isolated in various stereochemical forms ($EP_1$, (+)-$EP_2$, (−)-$EP_2$) and shown to be the chemical precursor of OC and 7,4′-dihydroxyflavonol (**4**). Concomitant with the formation of these novel compounds as major products, the formation of the "hydrated aurone" diastereoisomers ($Y_1$ and $Y_2$) (**5**) and 7,4′-dihydroxydihydroflavonol (**6**) were also observed with the purified peroxidase enzymes. Figure 1 summarises the interrelationship established [1–3] for these products. The enzymic reactions leading to the conventional type flavonoid compounds (**5**) and (**6**) were generally minor compared with chalcone peroxide (**2**) formation. However, in the presence of suitable additives, the latter pathway can be suppressed, leaving these minor compounds as the only significant products. This was in fact the situation in the original experiments carried out with crude cell-free extracts [2–4]. The biochemistry of the enzymic reaction leading to the major product (**2**) and observations relating to formation of the minor flavonoid products form the subject of this report.

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For simplicity, the terms chalcone, flavonol etc. will be used in the remainder of this paper to denote the particular 7,4′-dihydroxy compounds concerned.

## RESULTS

### *Purification of peroxidase from garbanzo seedlings*

Peroxidase was partially purified from cell-free extracts of garbanzo seedlings (*Cicer arietinum*) by successive acid treatment, acetone precipitation and column chromatography. Results are summarised in Table 1. Comparative studies were carried out with the purified garbanzo enzyme and commercial horseradish peroxidase (HRP).

### *Spectrophotometric monitoring of the reaction*

The oxidation of chalcone to EP compounds in the presence of enzyme and $H_2O_2$ was conveniently followed spectrophotometrically as a decrease in substrate *A* at 396 nm ($\lambda_{max}$ in 0.05 M Tris–HCl, pH 8). Product *A* was negligible under the same conditions. With horseradish peroxidase (HRP) the reaction curve was sigmoidal if the chalcone was preincubated with the buffer before enzyme and $H_2O_2$ were added to start the reaction. However, a hyperbolic reaction curve resulted when the reaction was started immediately after chalcone addition. The maximum reaction rate was also greater when the reaction curve was hyperbolic. When reaction rate was of interest, therefore, the reaction was started immediately after the addition of chalcone to the buffer. With peroxidase prepared from garbanzo seedlings (garbanzo enzyme) the curve for reaction at pH 8 was consistently hyperbolic.

### *pH-Activity profile*

Activity as a function of pH observed with chalcone

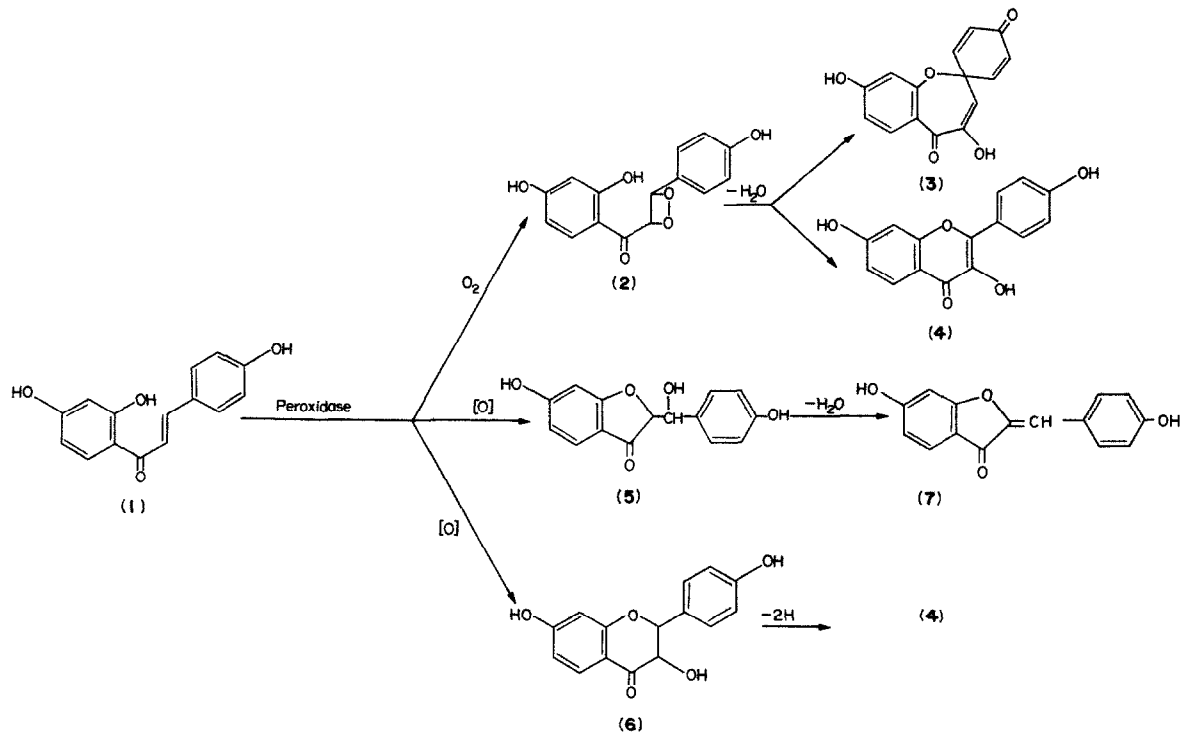



Fig. 1. Products of the enzymic reactions and their chemical transformation products.

Table 1. Purification of peroxidase from garbanzo seedlings

| | Volume (ml) | Concn (units/ml) | Total units ($\times 10^{-3}$) | Protein (mg/ml) | Sp act (units/mg) | Yield (%) | Purification |
|---|---|---|---|---|---|---|---|
| Cell-free | 57 | 2520 | 144 | 94 | 27 | 100 | 1 |
| Acid treated | 49 | 1960 | 96 | 8 8 | 223 | 67 | 8.3 |
| Acetone ppt. | 4 | 17600 | 70 5 | 34.5 | 510 | 49 | 19 |
| Sephadex G-75 | 34 | 1720 | 59 | 1 19 | 1445 | 41 | 54 |
| DEAE-Sephadex | 49.5 | 865 | 43 | 0011 | 77300 | 30 | 2880 |
| DEAE-Sephadex fraction 3 | 16 | 880 | | 0.004 | 204500 | | 7620 |

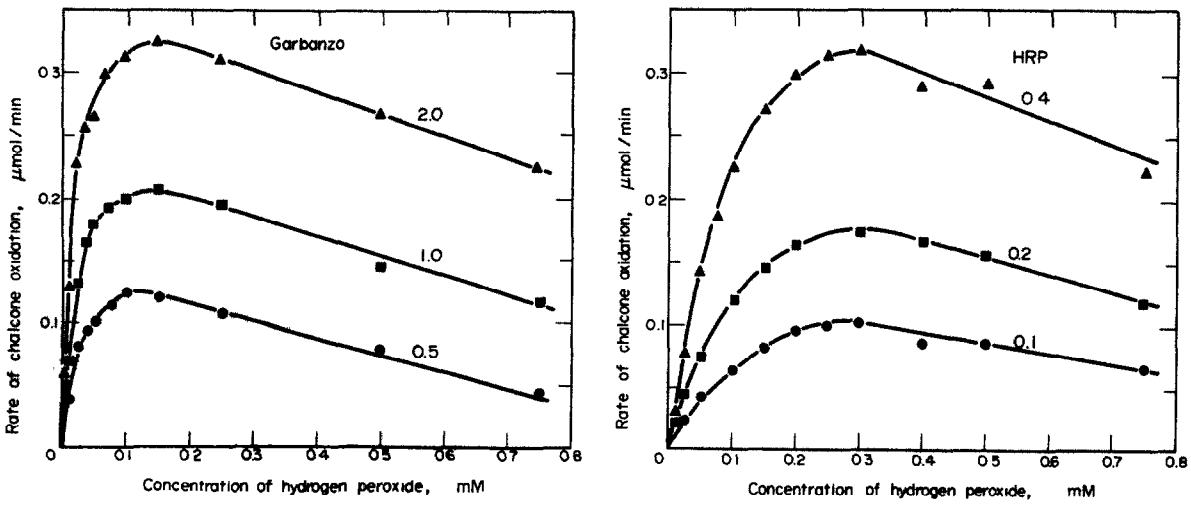



Fig. 2. Effect of $H_2O_2$ concentration on the rate of chalcone oxidation. The reaction mixture contained chalcone (0.09 μmol), $H_2O_2$ as shown and enzyme (units indicated by numbers on curves) in 0.05 M Tris buffer pH 8, total volume 2 ml, temperature 25°.

substrate for garbanzo enzyme and HRP showed definite optima at pH 7.5 and 7.9 respectively.

*Requirement for $H_2O_2$*

Virtually no reaction was observed when only enzyme and chalcone were incubated in buffer. Reaction occurred, however, upon addition of $H_2O_2$. This reaction rate as a function of the concentration of $H_2O_2$ added is recorded in Fig. 2 for HRP and garbanzo enzyme. The curves resembled substrate concentration curves with inhibition by higher substrate concentrations. Differences existed between the two enzymes, firstly in relation to the optimal concentration of $H_2O_2$ (Fig. 2) and secondly, in their activity towards chalcone per unit of activity assayed with guaiacol.

*Effect of chalcone concentration on reaction rate*

Typical substrate concentration curves were obtained when the chalcone concentration in the reaction mixture was increased from a low value with both garbanzo enzyme and HRP (Fig. 3). Both enzymes appeared to be saturated at chalcone concentrations of 40–50 μM.

*Oxygen consumption*

Manometric experiments showed that $O_2$ was consumed in stoichiometric amounts in the reaction (Table 2). With garbanzo enzyme the molar ratio of oxygem consumed to chalcone added was about 0.85 on average, while for HRP it was close to 1.0 (the lower ratio with garbanzo enzyme may be due to the incomplete consumption of chalcone since reaction mixtures decolorized completely only with HRP). Further, this stoichiometry was not influenced by the variation in $H_2O_2$ concentration and no strict stoichiometric requirement for this compound was evident. These features of the reaction were confirmed in additional manometric experiments with HRP. Thus oxidation of a fixed amount of chalcone (1.5 μmol) was always complete and matched by equimolar $O_2$ uptake when the $H_2O_2$ added was increased in steps from 0.22 to 3.1 μmol. The obligatory requirement for $O_2$ in the reaction was shown by the complete inhibition of chalcone oxidation to the peroxide product under anaerobic conditions in the presence of enzyme and stoichiometric amounts of $H_2O_2$.

*Consumption of $H_2O_2$*

While manometric results (Table 2) pointed to a stoichiometric requirement for $O_2$ rather than $H_2O_2$, the reaction rate was nevertheless affected by the $H_2O_2$ concentration in a manner compatible with this compound being a co-substrate (Fig. 2). However, further experiments demonstrated a catalytic rather than a stoichio-

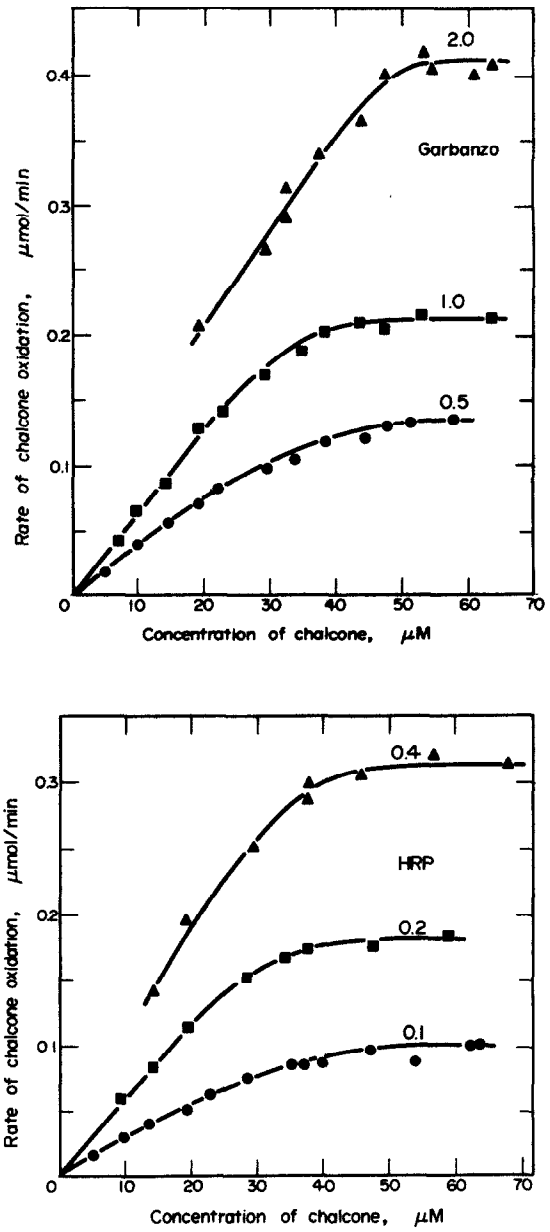



Fig. 3. Reaction rate as a function of chalcone concentration. The reaction mixture contained chalcone as shown, enzyme (units indicated by numbers on the curves) and $H_2O_2$ (0.3 μmole with garbanzo and 0.6 μmol with HRP) in 0.05 M Tris buffer pH 8, total volume 2 ml, temperature 25°.

Table 2. Oxygen consumption in the peroxidase-catalysed oxidation of chalcone

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| $H_2O_2$ (μmol) | 1.0 | 1.0 | 1.0 | 0.5 | 0.25 | 3.0 | 3.0 | 3.0 | 1 5 |
| Chalcone (μmol) | 2.15 | 1.90 | 1.52 | 1.16 | 0.90 | 2.66 | 2.47 | 1.87 | 0.90 |
| $O_2$ uptake (μmol) | 1.79 | 1.71 | 1.32 | 0.96 | 0.75 | 2.74 | 2.52 | 1.80 | 0 88 |
| $\frac{O_2 \text{ uptake}}{\text{chalcone}}$ | 0.83 | 0.90 | 0.87 | 0.83 | 0.84 | 1.03 | 1.02 | 0.96 | 0.98 |
| Enzyme (units) | | | | | | | | | |
| Garbanzo | 53 | 26 | 53 | 26 | 10 | — | — | — | — |
| HRP | — | — | — | — | — | 13 | 13 | 13 | 13 |

The reaction mixture contained chalcone, enzyme and $H_2O_2$ as shown in 4 ml total volume 0.05 M Tris buffer pH 8, temperature 25°. $O_2$ uptake was measured manometrically.

metric net consumption of $H_2O_2$ with HRP and garbanzo enzyme. In the complete oxidation of chalcone (0.37 or 0.27 or 0.18 μmol), catalysed by HRP (1 unit), the respective average net consumption of $H_2O_2$ was 0.02, 0.02 and 0.01 μmol and independent of the amount added in the range 0.2–0.65 μmol. Comparable results were obtained with garbanzo enzyme.

*Extent of reaction*

The composition of the reaction mixture had a marked effect not only on the rate of reaction as already noted, but also on the extent of chalcone consumption before the reaction virtually ceased. Only with certain reaction mixtures was chalcone consumption complete. The extent of reaction was sensitive to the concentration of each component under certain conditions. As the concentration of $H_2O_2$ increased, for example, the extent of reaction increased rapidly and became complete before being curtailed again at higher concentrations. The extent also increased with enzyme concentration. For example, in a reaction mixture which contained chalcone (0.37 μmol), $H_2O_2$ (0.2 μmol) and HRP in 2 ml Tris buffer pH 8, consumption of chalcone continued to about 45, 70 and 90% completion with enzyme additions of 0.05, 0.1 and 0.2 unit respectively.

*Effect of additives*

The effects of a range of additives on the rates of chalcone oxidation catalysed by HRP and garbanzo enzyme were assessed spectrophotometrically and are summarised in Table 3. Where reaction curves were sigmoidal, maximum rather than initial rates were used in calculating the values presented. Cyanide could not be used satisfactorily since a rapid chemical reaction was observed when KCN was added to chalcone in pH 8 buffer.

$Cu^{2+}$ and $Mn^{2+}$ were inhibitory, the latter strongly so when added at a concentration which approached that of the added chalcone. Progress curves were sigmoidal with $Cu^{2+}$ but not with $Mn^{2+}$. With HRP, the reaction showed a more pronounced lag phase but attained a greater rate at the higher compared with the lower $Cu^{2+}$ concentration. EDTA was slightly inhibitory. Diethyldithiocarbamate induced strongly sigmoidal progress curves, the very marked lag phase of which lasted for about 50 sec for the lower and about 150 sec for the higher concentration of the compound. Mercaptoethanol was also a very potent inhibitor but progress curves were not sigmoidal. The redogenic donors [5] (donors of which the free radicals exhibit reducing power), hydroquinone, catechol, pyrogallol and *p*-phenylenediamine, were all strongly inhibitory at a concentration similar to the chalcone. In the presence of the lowest concentration of hydroquinone, the reaction was still strongly inhibited but progressed without any lag. No stimulatory effect was recorded for the oxidogenic [5] donor, phenol.

Table 3. Effect of additives on the rate of enzymic oxidation of chalcone

| Additive | Final concn (mM) | Reaction rate as % of control | |
|---|---|---|---|
| | | Garbanzo enz. | HRP |
| $Cu^{2+}$ (as $CuSO_4$) | 1.0 | 34 | 54 |
| | 0.2 | 49 | 42 |
| $Mn^{2+}$ (as $MnCl_2$) | 0.2 | 3 | 4.5 |
| | 0.08 | 9 | 13 |
| EDTA* | 1.0 | 81 | 87 |
| SEDC‡ | 0.004 | 12 | 40 |
| | 0.002 | 48 | 60 |
| 2-Mercaptoethanol | 0.02 | 10 | 13 |
| | 0.008 | 25 | 30 |
| Hydroquinone | 0.1 | 0.3 | 0.4 |
| | 0.020 | 1.3 | 2.0 |
| | 0.002 | 14 | 16 |
| Catechol | 0.05 | 0.3 | 0.4 |
| Pyrogallol | 0.05 | 0.3 | 0.4 |
| *p*-Phenylenediamine | 0.05 | 0.1 | 0.2 |
| Phenol† | 0.1 | 100 | 92 |

Basic reaction mixtures contained, enzyme (garbanzo 1 unit, HRP 0.2 unit), chalcone (0.09 μmol–45 μm) and $H_2O_2$ (0.3 μmol with garbanzo and 0.6 μmol with HRP) in 0.05 M Tris buffer pH 8, total volume 2 ml, temperature 25°. Additives were freshly dissolved in $H_2O$ or EtOH at concentrations such that 5–25 μl aliquots were combined in the reaction mixtures. Enzyme and additive were mixed with buffer already at 25° and incubated for 2 min; chalcone was then added followed immediately by $H_2O_2$ to complete the system. * Disodium salt. † With this additive, the $H_2O_2$ added was cut to one-sixth of that given in the basic reaction mixtures above. Chalcone additive were mixed with buffer at 25°, then enzyme and $H_2O_2$ were added immediately. Results are expressed in terms of the appropriate new control rates. ‡ Diethyldithiocarbamate, sodium salt.

*Proportions of stereoisomeric enzymic products formed*

The proportions of $EP_1$ and $EP_2$, and of the two enantiomeric forms of $EP_2$ present in the enzymic reaction products under a variety of reaction conditions are presented in Table 4.

*$Y_1$, $Y_2$ formation*

In the course of the study on chalcone peroxide formation from chalcone, inspection of a large number of 2D-PC's of products of the reaction carried out under a range of concentrations of enzyme (both purified garbanzo peroxidase and HRP), chalcone and $H_2O_2$ showed that the 'hydrated aurone' stereoisomers, $Y_1$ and $Y_2$, were invariably formed as minor products.

$Y_1$, $Y_2$ coincide chromatographically with $EP_1$, $EP_2$ respectively, in virtually all solvent systems used. Alkali treatment of eluates from these two areas yield aurone and flavonol which are then separable on rechromatography. Quantitative estimates of $Y_1$, $Y_2$ could thus be

Table 4. Proportions of EP compounds in enzymic reaction product

| Reaction pH | Added chalcone (μmol) | Enzyme | Proportions in Products | |
|---|---|---|---|---|
| | | | $\frac{EP_1}{EP_2}$ | $\frac{(+)\text{-}EP_2}{(-)\text{-}EP_2}$ |
| 8.0 | 1.8 | HRP | 0.85 | 1.30 |
| 8.0 | 1.8 | G* | 0.81 | 1.30 |
| 8.0 | 3.6 | HRP | 0.81 | 1.12 |
| 8.0 | 3.6 | G | 0.60 | 1.13 |
| 7.0 | 1.8 | HRP | 0.80 | 0.92 |
| 7.0 | 1.8 | G | 0.80 | 0.94 |
| 7.0 | 3.6 | HRP | 1.00 | 0.93 |
| 7.0 | 3.6 | G | 0.69 | 0.93 |

* Garbanzo enzyme. Reaction mixtures contained, in a total volume of 4 ml 0.05 M Tris buffer (pH as above), chalcone (given above), enzyme (HRP 34 units, garbanzo 66 units) and $H_2O_2$ (2 μmol with HRP and 1 μmol with garbanzo enzyme). Reaction time was 3 min at room temperature and the higher chalcone concentration was aerated during this time. Products were extracted with $Et_2O$ after acidification of the reaction medium to pH 2–3.

obtained by fluorometric estimation of the aurone formed. Under typical conditions, the combined yield of $Y_1$ and $Y_2$ in the reaction accounted for about 1% of the chalcone reacted. In view of the labour involved, few quantitative measures of $Y_1$, $Y_2$ were actually made. In general, statements in this paper regarding amounts of $Y_1$, $Y_2$ are based on semi-quantitative visual assessment of 2D-chromatograms obtained under conditions where the EP compounds were largely transformed to OC (**6**) prior to the chromatographic step [1]. The $Y_1$, $Y_2$ spots then showed up in the chromatogram on keeping as the fluorescent aurone artefact (**7**).

*Effects of additives on the formation of $Y_1$, $Y_2$*

The effects of certain additives on the formation of $Y_1$, $Y_2$ were investigated (cf. Table 3). Redogenic donors such as hydroquinone and catechol inhibit $Y_1$ and $Y_2$ formation, together with formation of the main products $EP_1$, $EP_2$. Similarly, $Mn^{2+}$ was strongly inhibitory. On the other hand with diethyldithiocarbamate, found also to be an inhibitor of the main reaction, a relative increase in $Y_1$, $Y_2$ formation (about double that for the control) was noted. Parallel to the findings with main product formation, the formation of $Y_1$, $Y_2$ was also greatly reduced under anaerobic conditions.

In the original study [2,3] crude cell-free extracts catalysed the conversion of chalcone to $Y_1$ and $Y_2$ while no EP compounds (or OC) were observed. When the enzyme was purified by removal of soluble substances, two notable changes in the reaction occurred; decreased $Y_1$, $Y_2$ formation and increased chalcone disappearance due to major formation of EP compounds. The conclusion was drawn that co-factor(s) capable of inhibiting $EP_1$ and $EP_2$ formation and of promoting $Y_1$, $Y_2$ formation are present in crude cell-free extracts of garbanzo. Supernatant of boiled cell-free extracts was found indeed to have such effects. Fractionation experiments demonstrated that the compound(s) responsible was highly water soluble, non-ionic and non-extractable by organic solvents such as ether and ethyl acetate. The evidence indicated that the active compound(s) possessed properties similar to those of simple sugars.

*Dihydroflavonol formation*

The formation of dihydroflavonol (**6**) (garbanzol) from chalcone in trace amounts with cell-free enzyme preparation from garbanzo seedlings has been established earlier [2,4]. The question of whether formation of dihydroflavonol could be accounted for by peroxidase catalysis was checked in two ways. When chalcone [$^{14}$C] was used as substrate in the normal reaction catalysed by HRP enzyme, radioactive dihydroflavonol was isolated in about 0.5% yield (after addition of carrier). In the presence of hydroquinone, a specific inhibitor of peroxidase activity, radioactive dihydroflavonol formation in the cell-free enzyme preparation was found to be inhibited. These results led to the conclusion that dihydroflavonol is a definite, albeit very minor product of the peroxidase catalysed oxidation of chalcone.

*Chalcone substrate specificity*

Seven chalcones (**8–14**) in addition to 4,2′,4′-trihydroxychalcone (**1**) were tested as substrates in a peroxidase-catalysed reaction. Products were not isolated; only disappearance of the chalcone was followed spectrophotometrically. Control reactions, with enzyme either omitted or heat inactivated, were run. All chalcones except (**8**) and (**11**) underwent enzymic reaction. No difference was apparent in the specificity of HRP and garbanzo enzyme. No attempt was made to measure accurately differences in reaction rates between the chalcones since difficulty was experienced in purifying chromatographically the small amounts available of a number of these chalcones. The prepared compounds were contaminated with inhibitory impurities eluted from the chromatography paper despite the prior washing treatment.

None of the chalcones (**8–14**) reacted as rapidly as 4,2′,4′-trihydroxychalcone (**1**). Chalcone (**9**) reacted readily but at a rate *ca* 25% of that of (**1**). Another which reacted readily was (**14**), but the product in this case was extremely unstable and absorption rapidly reappeared at the absorption maximum of the chalcone. Chalconaringenin (**13**) reacted readily in the enzymic reaction and was also unstable in the reaction buffer. Considerably less effective as a substrate was chalcone (**12**) while (**10**) reacted but slowly.

These results suggested that a free phenolic hydroxyl group in the B ring, preferably in the *para* position, was required for activity in the enzyme catalysed reaction. It cannot be assumed however, that the enzymic reaction observed for the additional chalcones was necessarily equivalent to that undergone by isoliquiritigenin since only chalcone disappearance was followed.

## DISCUSSION

Comparative studies of garbanzo enzyme and HRP clearly demonstrate a uniformity of catalytic properties which establishes the garbanzo enzyme as a peroxidase. Thus the products of chalcone oxidation are identical for the two enzymes [1], $O_2$ is obligatory for the reaction and the effects of various additives (Table 3) on the rate of reaction are similar. Some quantitative differences are however apparent between the enzymes; the activity toward chalcone in relation to the peroxidatic activity toward guaiacol is greater for HRP by a factor of about 5 (Fig. 2); the garbanzo enzyme exhibits maximal reaction rates at a concentration of $H_2O_2$ about half of that similarly required by HRP; and the pH optimum for the garbanzo enzyme is also significantly lower than that for HRP. Gel electrophoresis showed both preparations to be mixtures of isoenzymes. Since the catalytic properties of isoenzymes of peroxidase can differ [6–9], the observed properties probably represent the weighted average of those of the component isoenzymes.

The results presented show that the peroxidase catalysed oxidation of chalcone (**1**) to the main product (**2**) utilizes molecular $O_2$ in equimolar amounts in the presence of $H_2O_2$ of which only a catalytic net consumption occurs. No other co-factors or additives are required. Thus the reaction may be summarised as follows:

$$RH_2\,(\text{chalcone}) + O_2 \rightarrow RH_2O_2 \qquad (1)$$

This would appear to place peroxidase in the novel role of being a dioxygenase [10], in contrast to its known oxidase activity in which molecular $O_2$ functions as electron acceptor in place of $H_2O_2$ [11]. Yamazaki [5,12–14] has proposed a generalised mechanism for per-

oxidase–oxidase activity which can be summarised by eqs. (2)–(5) below:

$$\text{Initiation} \quad YH_2 \xrightarrow{\text{Peroxidase-}H_2O_2} YH^{\cdot} + H_2O \qquad (2)$$

$$\text{Chain reaction} \quad YH^{\cdot} + O_2 \rightarrow Y + HO_2^{\cdot} \qquad (3)$$

$$YH_2 + HO_2^{\cdot} \rightarrow YH^{\cdot} + H_2O_2 \qquad (4)$$

$$\text{Net} \quad YH_2 + O_2 \rightarrow Y + H_2O_2 \qquad (5)$$

A catalytic amount of $H_2O_2$ is necessary to initiate the reaction by forming the substrate free radical $YH^{\cdot}$. Reaction with molecular $O_2$ is then possible, producing the perhydroxyl radical which in turn can oxidise a second molecule of substrate to regenerate the free radical. An essentially non-enzymic chain reaction is thereby established. Coupled to this oxidatic activity, non-specific oxygenation of aromatic rings (hydroxylation) is sometimes possible, with perhydroxyl radical being the probable oxygenating species [15,16].

Analogous to the oxidatic mechanism above, we propose here a non-enzymic radical chain reaction mechanism for the dioxygenation of chalcone ($RH_2$) which is consistent with all of the salient biochemical features established in this work. This scheme is summarised in eqs. (6)–(8):

$$\text{Initiation} \quad RH_2 \xrightarrow{\text{Peroxidase-}H_2O_2} RH^{\cdot} + H_2O \qquad (6)$$

$$\text{Chain reaction} \quad RH^{\cdot} + O_2 \rightarrow RHO_2^{\cdot} \qquad (7)$$

$$RH_2 + RHO_2^{\cdot} \rightarrow RH_2O_2 + RH^{\cdot} \qquad (8)$$

$$\text{Net} \quad RH_2 + O_2 \rightarrow RH_2O_2 \qquad (1)$$

The scheme differs from that of Yamazaki (eqs. (2)–(4)) only in that chalcone peroxide $RH_2O_2$ and its free radical $RHO_2^{\cdot}$ replace $H_2O_2$ and $HO_2^{\cdot}$ respectively in the sequence. As in the general oxidatic reaction, the chain propagation steps are taken to be non-enzymic, so that in essence peroxidase can still be regarded as functioning enzymatically in its classical peroxidatic mode [11]. The net overall result however is dioxygenation instead of dehydrogenation, and represents a 4-equivalent instead of 2-equivalent oxidation of substrate.

The existence of the enzymic product (**2**) in all of its possible stereoisomeric forms [1] and in comparable amounts (Table 4), provides full support for the non-enzymic reaction scheme presented in Eqs. (7)–(8). In Fig. 4 chemical structures for the intermediate chalcone oxidation products implicated in the scheme have been formulated and a mechanistically acceptable rationalisation for the postulated reaction sequence given. In this scheme, formation of the chalcone free radical $RH^{\cdot}$ is envisaged as originating from the 4-hydroxyl of the B ring. The requirement of this functional group for activity is strongly suggested from the preliminary results of the substrate specificity studies. Mesomeric shift of the radical centre allows initial non-stereospecific oxygenation at the α-carbon. Subsequent tautomerism results in oxygen ring closure at the β-carbon atom which is again non-stereospecific. Judging from the values (~ 1) found for the ratios of different stereoisomers formed (Table 4), it can be concluded that in each of the two oxygenation steps, formation of either of the epimeric forms is of about equal probability.

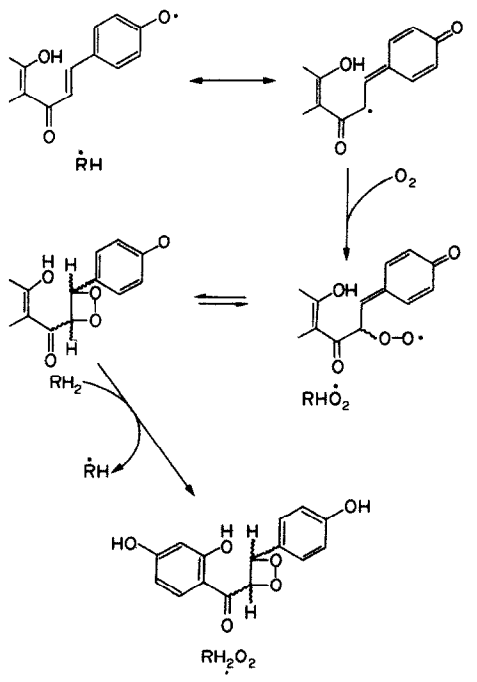
There are a number of strong similarities in the oxygenation of chalcone with the oxidation of indole-3-acetic acid (IAA) catalysed by peroxidase, another 4-equivalent proposed a mechanism in which IAA radical reacts with molecular $O_2$ to form 3-methylene oxindole and indole-3-aldehyde as main products through several non-enzymic steps. Results from more recent studies are in accord with this view [14,19].

In addition to the chalcone peroxide (**2**), the peroxidase catalysed oxygenation of chalcone also produces the 'hydrated aurone' $Y_1$, $Y_2$ (**5**) and dihydroflavonol (**6**) as minor products. These differ from the main product in being monooxygenation products of chalcone, representing 2-equivalent oxidation of substrate. A clue to the probable origin of these minor products may lie in the finding that in the presence of additives such as diethyldithiocarbamate (SEDC) and cell-free supernatant, $Y_1$, $Y_2$ production is proportionately increased even though chalcone peroxide formation (and overall chalcone consumption) is greatly reduced. In terms of the radical chain mechanism proposed in eqs. (7)–(8), inhibition of chalcone peroxide formation will be effected by reactions which can break the chain sequence. The organic compounds studied which are strong inhibitors (Table 3) are all good electron donors or strong reducing agents, as are mercaptoethanol and SEDC and presumably also the unknown co-factor(s) in crude cell-free extracts. These compounds ($AH_2$) presumably function as chain-breakers in the reactions leading to chalcone peroxide by scavenging of free radicals derived from the chalcone

$$AH_2 + RH^{\cdot} \rightarrow AH^{\cdot} + RH_2 \qquad (9)$$

$$AH_2 + RHO_2^{\cdot} \rightarrow AH^{\cdot} + RH_2O_2 \qquad (10)$$

If, in addition, we postulate that these same inhibitors can also reduce, to various degrees, any chalcone peroxide that does get formed under these conditions, then 2-equivalent oxidation products such as $Y_1$, $Y_2$ and dihydroflavonol could be formed as reduction products of the chalcone peroxide. This possible reduction route



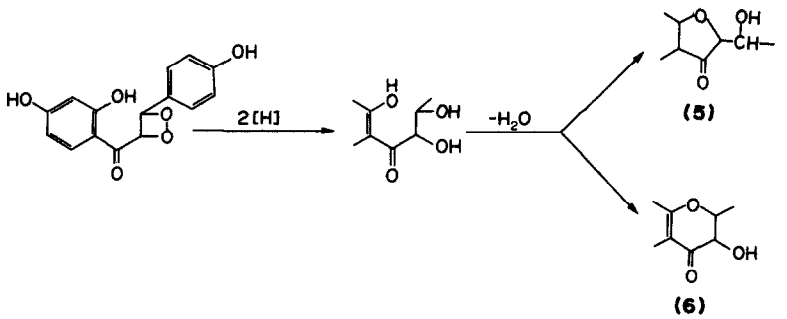



Fig. 5. Possible derivation of hydrated aurone, and dihydro-flavonol.

to $Y_1$, and $Y_2$ and dihydroflavonol, ($RH_2O$), is represented stoichiometrically below in Eq. (11)

$$RH_2O_2 + AH_2 \rightarrow RH_2O + H_2O + A \qquad (11)$$

and is illustrated chemically in the scheme in Fig. 5. A balance of the two possible effects of an inhibitor postulated above would produce the 2 types of overall effects actually observed, viz. either all products are inhibited, or EP compounds (and hence OC and flavonol) are inhibited whilst $Y_1$, $Y_2$ may be proportionately increased. The formation of trace amounts of $Y_1$, $Y_2$ and dihydroflavonol in systems where purified enzymes were used must mean that some reduction of chalcone peroxide was still possible in these systems in the absence of externally added reducing agents.

The *in vivo* significance, if any, of the enzymic reactions described by this paper, is a question of great interest. Although OC (3), the main transformation product of the *in vitro* reaction with purified peroxidase, does not itself represent a recognised class of flavonoid product, the fact that in the presence of other substances and under suitable reaction conditions products of conventional type, namely flavonol, dihydroflavonol and aurone, can be formed via peroxidase catalysis, is possibly of significance. These more complex latter situations could more likely be representative of the conditions obtaining in the intact cell where peroxidase activity would be envisaged to be influenced by other cell constituents and be under regulation and control. Irrespective of the true biogenetic significance, the enzyme reactions reported in this paper serve as novel and interesting examples of the type of oxidation reactions feasible for the generation of other classes of flavonoids from chalcones.

## EXPERIMENTAL

*Partial purification of peroxidase from garbanzo seedlings.* All operations were carried out at 0–4°. Seedlings were ground in 0.01 M Tris pH 7.5 (about 7 ml/10 g seedlings) in a Waring Blendor and the homogenate squeezed through cheesecloth. Expressed liquid was centrifuged at 5000 *g* for 30 min to yield cell-free preparation (about 7.5 ml/10 g seedlings). Protein was precipitated by acidification to pH 4.5, removed, by centrifugation (5000 *g* for 10 min) and supernatant readjusted to pH 7.5. $Me_2CO$ was added to 50% final concn, the precipitated protein was recovered by centrifugation (5000 *g* for 20 min), resuspended in 0.05 M Tris pH 7.5 (0.6 ml/10 g seedlings) and centrifuged. The supernatant was chromatographed on Sephadex G-75 (3 × 36 cm) in 0.05 M Tris pH 7.5. Activity was recovered in fractions 15–18 inclusive (fraction vol 8.5 ml). Enzyme (5 ml sample) was further purified, either immediately or after storage in freeze dried form, by chromatography on DEAE Sephadex A-50 (1 × 10 cm) with 0.05 M Tris pH 7.5. Activity was recovered in fractions 3–6 inclusive (about 2.5 ml each) just ahead of a small protein peak. Active fractions were kept at 0–4°. The most active fraction (3) was used as the source of garbanzo enzyme. Polyacrylamide gel electrophoresis indicated that several isoenzymes were present.

*Assay of peroxidase.* Peroxidation of guaiacol to coloured products monitored at 470 nm was used [20]. The reaction vol of 2 ml in 0.05 M Tris–HCl pH 7.5, contained guaiacol (25 μl of 26.7 mM soln in $H_2O$), $H_2O_2$ (10 μl of 26.7 mM soln in $H_2O$) and enzyme (usually 5–25 μl in buffer). Addition of $H_2O_2$ started the reaction which was run at 25°. Enzyme was adjusted to give an activity of 50–100 units. The unit of activity used is that required to produce an increase of 0.01 in *A* at 470 nm in 5 min in the above assay. Maximum rates taken from the progress curves were used to calculate activity. With garbanzo enzyme these were always initial rates but with HRP a short lag phase preceded the phase of linear maximum rate.

*Enzymic reaction of chalcone.* For spectrophotometric observation the reaction was run in a vol of 2 ml in 1 cm path length cells, at 25°. The reaction was started by addition of $H_2O_2$ (in 10 μl) immediately after addition of chalcone (in 25 μl of EtOH) and enzyme (in 10 μl of buffer). Reaction curves were recorded at $\lambda_{max}$ for chalcone (396 nm in pH 8 buffer). Rates were calculated using an $E^{1\%}_{1cm}$ value for chalcone, as the monohydrate, of $1.07 \times 10^3$. In the determination of the pH-activity profile, values of $\lambda_{max}$, and $E^{1\%}_{1cm}$ were determined for each buffer pH.

*Anaerobic reactions.* The reaction was run in Thunberg tubes or similar spectrophotometric cuvettes. Chalcone and enzyme were mixed with buffer in the tube and $H_2O_2$ in a small vol of $H_2O$ was placed in the side arm. The assembly was then evacuated for 10 min. $O_2$-free $N_2$, provided by passing a slow stream of commercial $O_2$-free $N_2$ through a tube packed with freshly reduced Cu turnings and heated to 400°, was then introduced and the system re-evacuated for a short time, the cycle being repeated ×3. The tube was then filled with $N_2$ to atmospheric pressure and sealed. The contents of the assembly were mixed at 25° and incubated. For spectrophotometric observation, the complete anaerobic reaction mixture contained chalcone (0.73 μmol), HRP (1 unit) and $H_2O_2$ (0.55–1.5 μmol) in 0.05 M Tris pH 8.5, total vol 3 ml. A slight consumption of chalcone often observed immediately after mixing was attributed to traces of residual $O_2$. Further $H_2O_2$ (0.88 μmol) added to the stable anaerobic system produced no change in chalcone *A* but subsequent aeration produced immediate consumption of chalcone. Where products were observed chromatographically, chalcone (1.46 μmol) and HRP (200 units) were added to 4 ml of 0.05 M Tris pH 7.0 in a Thunberg tube and $H_2O_2$ (4.0 μmol) in 0.1 ml $H_2O$ was introduced under anaerobic conditions from the side arm. After incubation for 30 min at 25°, hydroquinone (4 μmol in EtOH) was added rapidly and mixed with the contents

of the tube to prevent aerobic reaction prior to solvent extraction [1]. (Aerobic controls displayed complete chalcone oxidation in less than 30 sec.)

*Manometry.* Standard procedures [21] were employed with the reaction mixtures given in Table 2. The concn of chalcone stock soln (diNa salt dissolved in pH 8 buffer) was determined spectrophotometrically before each manometric run since significant isomerization to flavanone occurred.

*Estimation of $H_2O_2$.* Concentrated stock soln, *ca* 30% $H_2O_2$, was standardized by titration against $KMnO_4$ in the presence of $H_2SO_4$. Residual $H_2O_2$ in the enzymic reaction mixture was estimated by coupling to the stoichiometric peroxidation of guaiacol (product monitored at 470 nm). Absorbance generated from guaiacol by a given amount of $H_2O_2$ was diminished in the presence of products of chalcone oxidation. Therefore, to construct a calibration curve the chalcone reaction was first run in the cuvette using the minimum fixed addition of $H_2O_2$ needed for complete reaction; standard amounts of $H_2O_2$ were then added along with guaiacol and extra enzyme (see below) and *A* at 470 nm determined. The calibration curve was obtained when these values were corrected for the uniform increment contributed by $H_2O_2$ remaining from the chalcone reaction. To estimate net $H_2O_2$ consumption in the chalcone reaction, residual $H_2O_2$ was determined with guaiacol and subtracted from that added to start the chalcone reaction. Estimation of $H_2O_2$ was performed rapidly (*A* at 470 nm unstable) upon addition of HRP (400 units in 5 $\mu$l) and guaiacol (0.65 or 1.30 $\mu$mol in 5 $\mu$l when total $H_2O_2$ was below or above 0.4 $\mu$mol respectively). The method was suitable in the range 0–1 $\mu$mol $H_2O_2$.

*Determination of proportions of EP compounds in reaction products.* Aliquots of the EP product from each reaction condition (Table 4) were chromatographed in 0.5% HOAc at 4° for 6 hr to separate $EP_1$ and $EP_2$ and for 16 hr to resolve (+)-$EP_2$ and (−)-$EP_2$. The EP compounds were detected upon limited UV irradiation of the wet chromatograms [1], excised and eluted for 30 min in 85% EtOH. Relative quantities of the EP compounds were directly obtained from the *A* of the eluates at 275 nm, since the $E^{1\%}_{1cm}$ values at this wavelength were similar ($EP_1$ 480, $EP_2$ 495).

*Purification and enzymic reaction of chalcones* (8)–(14). Chalcones (8–14) used were mostly available in only very small amounts from previous work [22]. About 1 mg of each compound, except chalconaringenin (**13**), was chromatographed successively on washed Whatman 3 MM paper in the solvents $C_6H_6$–HOAc–$H_2O$ (125:72:3), 30% HOAc and 30% *iso*PrOH. Paper was batch washed in 5% HOAc, rinsed in $H_2O$, dried and as single sheets irrigated with 85% EtOH for about 24 hr. Each compound was finally eluted in 85% EtOH. Reaction mixtures contained a chalcone (about 0.09 $\mu$mol for each compound), enzyme (HRP, 0.2 or 34 units, garbanzo, 1.0 or 25 units) and $H_2O_2$ (0.6 $\mu$mol with HRP and 0.3 $\mu$mol with garbanzo enzyme) in 0.05 M Tris pH 8.0, 2 ml total vol at 25°. The higher enzyme activities were used with all chalcones except (**1**) and (**8**).

*Effects of additives on $Y_1$ and $Y_2$ formation.* The reaction mixture contained: chalcone (1.8 $\mu$mol), enzyme (95 units), $H_2O_2$ (8 $\mu$mol) and additive (4.0 $\mu$mol—see below) in 4 ml total vol of 0.05 M Tris pH 8.5 at room temp. Additive (hydroquinone, catechol, $Mn^{2+}$ and Na diethyldithiocarbamate, singly) was combined with enzyme in buffer and 2 min later chalcone was added followed by $H_2O_2$. After incubation the mixture was acidified to pH 7.5 prior to extraction of the product into $Et_2O$. Qualitative information on $Y_1$ and $Y_2$ formation in the reaction was obtained after 2-D chromatographic separation of the products when overlapping EP compounds were sufficiently weak for the fluorescent aurone artefact to be visible after storage of the chromatograms.

*$Y_1$, $Y_2$ promoting and OC-inhibiting factor(s) in cell-free garbanzo enzyme.* Cell-free enzyme [2] was heated at 100° for 15 min, cooled to room temp and centrifuged to provide a supernatant devoid of peroxidase enzyme activity. This supernatant was subjected to fractionation by solvent extraction, ion exchange (both cation and anion), resin partition and PC in aq solvents. Fractions were assayed on the basis of their capacity to inhibit chalcone consumption on incubation. The assay reaction mixture contained the minimum of $H_2O_2$ needed for the reaction to go to completion in the absence of inhibitor; any significant inhibition by fractions was then readily detected visually by persistence of the chalcone colour.

*Evidence for dihydroflavonol formation in the HRP reaction.* The reaction mixture contained chalcone (carbonyl-$^{14}$C) [4] (0.9 $\mu$mol), HRP (5 units) and $H_2O_2$ (2 $\mu$mol) in 4 ml total vol Tris pH 7.5. In the control, enzyme in buffer was heated (15 min, 100°) and allowed to cool to room temp before the other components were added. After the rapid reaction in the complete system at room temp, the products were extracted directly into $Et_2O$ and chromatographed in 2-D, with carrier garbanzol (50 $\mu$g) present. Autoradiography showed the clearly resolved garbanzol spot to be weakly active in the enzyme run. Counts of the eluted garbanzol on planchettes gave activities of 1270 and 450 cpm for the enzyme and control runs respectively. Sp act of chalcone was 727 cpm/$\mu$g (23.5% counting efficiency).

*Inhibition of dihydroflavonol formation in cell-free systems.* The reaction mixture contained cell-free enzyme (4 ml), hydroquinone (5 mM final concentration in 0.1 ml), and radioactive chalcone (3,5-$T_2$) [4] (0.9 $\mu$mol) in pH 7.5 Tris, 5 ml total vol. The inhibitor was mixed with the enzyme before addition of chalcone and the mixture was incubated for 20 min at 37°. Hydroquinone was omitted from the control with both active and heat-inactivated (15 min, 100°) enzyme. Products were $Et_2O$ extracted from the pH 7.5 buffer after removal of protein and subjected to 2-D chromatography in the presence of carrier garbanzol. The garbanzol spot was eluted and activity determined by liquid scintillation counting. Total activity in the garbanzol spot was calculated as 3680, 155 and 92 cpm respectively for active enzyme with no inhibitor, with hydroquinone, and boiled enzyme with no inhibitor. Sp act of the chalcone was $1.71 \times 10^4$ dpm/$\mu$g.

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